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# DEVELOPMENT OF A CULTURE SYSTEM FOR BOVINE LUTEAL CELLS: EXAMINATION OF LUTEOTROPIC AND LUTEOLYTIC MECHANISMS

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EXAMINATION OF LUTEOTROPIC AND LUTEOLYTIC MECHANISMS

*University of New Hampshire*

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DEVELOPMENT OF A CULTURE SYSTEM FOR  
BOVINE LUTEAL CELLS: EXAMINATION OF  
LUTEOTROPIC AND LUTEOLYTIC MECHANISMS

BY

Joy Lee Pate  
B.S., University of Delaware, 1979

DISSERTATION

Submitted to the University of New Hampshire  
in Partial Fulfillment of  
the Requirements for the Degree of

Doctor of Philosophy  
in  
Zoology

May, 1983



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## LIST OF ABBREVIATIONS

1. ACAT = acyl coenzyme A:cholesterol acyltransferase
2. ACTH = adrenocorticotropic hormone
3. cAMP = adenosine 3',5'-monophosphate
4. CEH = cholesterol ester hydrolase
5. CL = corpus luteum
6. CT = cholera toxin
7. dbcAMP = dibutyryl adenosine 3',5'-monophosphate
8. FKN = forskolin
9. FSH = follicle stimulating hormone
10. GTP = guanosine 5'-triphosphate
11. hCG = human chorionic gonadotropin
12. HDL = high density lipoprotein
13. HEPES = N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
14. HMG-CoA reductase = 3-hydroxy-3-methylglutaryl coenzyme A reductase
15. INDO = indomethacin
16. LDL = low density lipoprotein
17. LH = luteinizing hormone
18. MgATP = magnesium adenosine 5'-triphosphate
19. NADPH = nicotinamide adenine dinucleotide phosphate (reduced)
20. 18-OH-DOC = 18-hydroxydeoxycorticosterone
21. P<sub>4</sub> = progesterone
22. PG = prostaglandin

## ABSTRACT

### DEVELOPMENT OF A CULTURE SYSTEM FOR BOVINE LUTEAL CELLS: EXAMINATION OF LUTEOTROPIC AND LUTEOLYTIC MECHANISMS

by

JOY LEE PATE

University of New Hampshire, May, 1983

The function of bovine luteal cells in tissue culture was examined. Corpora lutea from regularly cycling dairy cows were dissociated with collagenase and cultured in Ham's F-12 medium with or without serum. The serum-free medium was supplemented with insulin, transferrin and hydrocortisone. Addition of LH to the serum-containing medium did not increase  $P_4$  production. When the luteal cells were cultured in serum-free medium, LH produced an increase in  $P_4$  during the first 24 hours of culture. The responsiveness of the cells to LH then declined, and remained low until approximately day 7 of culture, at which time the cells regained their ability to respond to LH. The luteal cells were responsive to dbcAMP in both serum-containing and serum-free medium. These results indicate that the presence of serum in the cell culture medium inhibits the responsiveness of luteal cells to LH at a step prior to the increase in cellular cAMP.

The addition of either low density or high density lipoprotein to

the serum-free cultures produced a 150-260% increase in  $P_4$  production without inhibiting the LH response. In the presence of lipoproteins,  $^{14}C$ -acetate incorporation into sterols and steroids was greatly decreased. These experiments suggest that cultured bovine luteal cells can use serum lipoproteins as a source of cholesterol to increase  $P_4$  synthesis, and the lipoproteins can influence endogenous cholesterol metabolism.

The long-term effects of  $PGF_{2\alpha}$  on luteal function were examined using this cell culture system.  $PGF_{2\alpha}$  has either no effect on basal  $P_4$  production, or is slightly stimulatory. During the first 24 hours of culture,  $PGF_{2\alpha}$  has no effect on LH-, cholera toxin-, or forskolin-stimulated  $P_4$ . However, after day 1,  $PGF_{2\alpha}$  inhibits the agonist-induced increases in  $P_4$ , resulting in levels of  $P_4$  that are not different from controls.  $PGF_{2\alpha}$  is also capable of blocking indomethacin-stimulated increases in  $P_4$  production. These findings indicate that although  $PGF_{2\alpha}$  does not decrease basal steroidogenesis, it is able to block agonist-induced increases in  $P_4$  after day 1. The site of action of  $PGF_{2\alpha}$  is beyond the LH receptor, and is either at, or beyond, the adenylate cyclase molecule.

## INTRODUCTION

The bovine corpus luteum has been extensively studied in vivo and in vitro; however, most in vitro studies have involved short-term incubations of either luteal tissue slices or dissociated luteal cells. Although these systems exclude the influence and interference of endogenous hormones which are present in vivo, they cannot be used to evaluate long-term effects of hormones on luteal function. Cultured granulosa cells from the mare (Channing, 1966), pig (Schomberg, 1967), cow (Cirillo et al., 1969), human (Channing, 1969), rhesus monkey (Channing, 1970) and rat (Crisp and Denys, 1975; Erickson and Hsueh, 1978; Centola, 1979) are commonly used for long-term in vitro studies. However, there are few reports of dissociated luteal cells grown in tissue culture. Gospodarowicz and Gospodarowicz (1972) successfully cultured enzymatically dissociated bovine luteal cells; Stoklosowa and Stadnicka (1973) have grown porcine and rat luteal cells, and recently Goldsmith et al. (1981) reported a culture system for human luteal cells. Unfortunately, these systems have not been extensively employed to study the control of luteal steroidogenesis in vitro.

The presence of serum in the cell culture medium inhibits the induction of LH receptors by FSH in cultured rat granulosa cells (Erickson et al., 1979), as well as the ability of FSH to stimulate progesterin synthesis (Orly et al., 1980). Also, in cultured rat granulosa cells, Schuler et al. (1979) found that serum lipoproteins will increase steroid synthesis. The inhibitory effect of serum and the

stimulatory effect of lipoproteins have not been previously reported with cultured luteal cells.

Studies concerning the effects of  $\text{PGF}_{2\alpha}$  on bovine luteal tissue have produced conflicting results between in vivo and in vitro models. Although  $\text{PGF}_{2\alpha}$  consistently induces luteal regression in cows (Inskeep, 1973), in vitro incubations of luteal tissue preparations with this prostaglandin result in a stimulation of progesterone production (Speroff and Ramwell, 1970; Hixon and Hansel, 1979). This conflict may be due to the fact that the in vitro studies have been short-term (acute) in nature. Behrman (1979) reported that a rapid action of  $\text{PGF}_{2\alpha}$  on rat luteal tissue is an inhibition of LH-activated adenylate cyclase, but Jordan (1981) provided evidence to indicate that  $\text{PGF}_{2\alpha}$  produces a lesion in the steroidogenic pathway beyond the formation of cAMP. The exact nature of  $\text{PGF}_{2\alpha}$ -induced luteolysis, especially in the bovine species, remains to be elucidated.

The present studies were undertaken to determine the optimal conditions for the culture of bovine luteal cells. The influence of serum and serum-derived lipoproteins on luteal function were examined, and, using this cell culture system, the long term in vitro effects of  $\text{PGF}_{2\alpha}$  on progesterone production have been investigated.

## REVIEW OF THE LITERATURE

The ovaries of domestic mammals are located in the abdominal cavity and serve both a gametogenic function and an endocrine function. A single layer of cuboidal cells called the germinal epithelium covers the ovary and the tunica albuginea is found directly beneath this layer. The ovarian cortex is composed of interstitium, follicles, and corpora lutea. Unlike the laboratory animals (i.e. rats, rabbits, mice), the large domestic animals lack large amounts of steroid-secreting interstitial tissue, leaving ovarian steroidogenesis to occur only in the follicular and luteal compartments.

Ovarian follicles may be classified as 1) primary: an oocyte surrounded by a single layer of granulosa cells, 2) secondary: an oocyte surrounded by two or more layers of granulosa cells, or 3) tertiary: a follicle in which an antrum is present. First identified in 1672 by Regnier de Graaf (Asdell, 1969), the tertiary, or Graafian follicle is the most steroidogenically active follicle and is the most responsive to gonadotropins. The development from a primary to a tertiary follicle requires the interaction of both polypeptide and steroid hormones to initiate follicular growth and increase steroid biosynthesis (reviewed by Richards, 1980). The synthesis of estradiol by the Graafian follicle is accomplished by the coordination of the two cell types that comprise the follicle wall. The avascular granulosa cells line the antrum and are bathed in follicular fluid. The vascular theca interna cells surround the follicle and are separated from the granulosa cells by a basement membrane. In 1959 Falck provided the first evidence that these

two cell types act jointly to synthesize estrogen. Since that time more direct biochemical data has accumulated to support the "two-cell type" theory of estrogen synthesis (reviewed by Dorrington, 1977; Richards, 1980; and Hillier, 1981). Briefly, this two-cell theory proposes that androgen is synthesized from cholesterol in the theca interna and that a gonadotropin, luteinizing hormone, increases this conversion. This androgen is then transported across the basement membrane to the granulosa cells, where, under the direction of follicle stimulating hormone it is aromatized to estrogen.

#### Luteinization

As the mature follicle nears ovulation transformations begin to prepare the theca and granulosa cells for luteinization. One of the early events leading to follicular luteinization is the increase in numbers of LH receptors on the granulosa and theca cells (reviewed by Channing et al., 1980). FSH is responsible for the induction of LH receptors, and this action of FSH is mediated by the second messenger cAMP (Nimrod, 1981a; Knecht et al., 1981). Although the granulosa cells are able to bind LH at this time, they are not able to respond to the gonadotropin, probably because of the presence of a follicular fluid inhibitor (Channing et al., 1980). Shemesh (1979) has reported that follicular fluid obtained from mid-cycle follicles inhibits progesterone synthesis by cultured bovine granulosa and theca cells, but preovulatory follicular fluid does not have this effect.

Once ovulation has occurred, complete morphological and biochemical luteinization may take place. In the cow, both the theca interna and granulosa cells contribute to the formation of the corpus luteum (Gier and Marion, 1961; Donaldson and Hansel, 1965b). Immediately after ovula-



tion the follicle collapses forcing the stratum granulosum into many folds, each with a core of theca interna. The basement membrane breaks down, the granulosa cells enlarge, and by day 4 post-ovulation the folds completely fill the cavity. Theca interna cells migrate along the capillaries to mix with the granulosa cells. Gier and Marion (1961) have reported that the granulosa cells hypertrophy to form large luteal cells and the smaller, more irregular cells are derived from the theca. Donaldson and Hansel (1965b) agree that the granulosa develop into large, functioning luteal cells by day 4, but contend that further growth of the CL occurs by division and enlargement of theca interna-derived cells, possibly contributing to a second population of large luteal cells. Although the exact origin of the large and small luteal cells remains uncertain, it is now apparent that these two cell sizes may function differently in the mature CL. It appears that the large luteal cells are capable of higher levels of progesterone synthesis, while the small luteal cells are more responsive to gonadotropin stimulation (Ursely and Leymarie, 1979; Koos and Hansel, 1981). This is also true in the ovine (Fitz et al., 1982) and pregnant sow (Lemon and Loir, 1977) CL, and there may be a cooperative interaction between the two cell sizes resulting in increased steroidogenesis (Lemon and Mauleon, 1982).

During luteinization the cytoplasmic:nuclear ratio is increased in both theca and granulosa cells, there is a loss of rough endoplasmic reticulum and an increase in smooth endoplasmic reticulum. Increased lipid droplets are found in the cytoplasm, and the mitochondria change from possessing a lamelliform type of cristae to a highly developed villiform type (Channing et al., 1980).

Concurrent with the morphological transformation of granulosa and theca cells to mature luteal cells, the steroid biosynthetic machinery is altered from the production of estradiol to the production of large quantities of progesterone. The bovine CL does not secrete estradiol, and the luteinization of bovine granulosa cells results in a loss of the ability of these cells to aromatize androgens to estrogens (Henderson and Moon, 1979), even though the luteal cells have a much greater steroidogenic capacity than granulosa or theca cells. In the pig, luteal mitochondria are larger in size, have increased cytochrome P-450 and are more active in steroidogenesis than follicular mitochondria (Campbell et al., 1980). Also, Dimino (1977) reported that during luteinization of porcine granulosa cells the mitochondria gain the ability to convert pregnenolone to progesterone, but this is probably not the case in the bovine CL.

The increased steroidogenic capacity during luteinization is reflected in changing levels of  $P_4$  in the developing CL. On day 2 of the estrous cycle luteal  $P_4$  is approximately 7ug/g tissue, then increases to 25ug/g on day 4 and 46 ug/g on day 11. This high level is maintained until around day 20 when luteal  $P_4$  drops back to 25ug/g and continues to decrease to approximately 5ug/g on day 0 (Hafs and Armstrong, 1968). The bovine CL also synthesizes  $20\beta$ -dihydroprogesterone, a metabolite of progesterone which follows the same pattern as  $P_4$  but at much lower levels (1-10 ug/g tissue). The CL increases in weight from 360 mg (day 2) to about 6 g (day 11), then gradually declines (Hafs and Armstrong, 1968).

#### Luteotropism

A luteotropic substance is one which maintains a CL in morpholog-

lcal integrity and secreting normal amounts of progesterone. The luteotropic substance varies among species and may consist of a single hormone or a complex of hormones. In most cases the luteotropin is a pituitary-derived hormone; however, estrogen is also luteotropic in some species. Normal luteal function requires prolactin, FSH and trace amounts of LH in the hamster (Greenwald, 1967; Greenwald, 1973); LH and prolactin in the ewe (Denamur et al., 1973); and prolactin, LH and estrogen in the rat (Hilliard, 1973).

In contrast, LH alone appears to be the luteotropin in the cow. Mason et al. (1962) reported that LH, but not prolactin, was able to stimulate  $P_4$  synthesis by slices of bovine luteal tissue incubated in vitro, and Armstrong and Black (1966) also found LH to be effective in CL obtained on days 1-18 post estrum. In addition, in vivo studies support the conclusion that LH is the single luteotropin in the bovine. In helpers, the injection of oxytocin on days 2-6 of the estrous cycle results in a marked shortening of the diestral period (Armstrong and Hansel, 1959). However, the simultaneous administration of LH (but not prolactin, growth hormone or FSH) overcomes the luteolytic effects of oxytocin (Donaldson et al., 1965; Hansel, 1966). Also, a single injection of LH on day 16 of the cycle is able to prolong the lifespan of the normal CL, resulting in an increased cycle length of 34 days (normal cycle=20-22 days) (Donaldson and Hansel, 1965a).

While it is now generally accepted that LH is the luteotropin in the bovine, there are other endogenous factors, such as catecholamines, that are able to stimulate  $P_4$  synthesis in bovine (Condon and Black, 1976), ovine (Jordan et al., 1978) and rat (Ratner et al., 1980) luteal tissue in vitro. However, the physiological role of catecholamines in  $P_4$

synthesis is uncertain.

### LH Receptor

Much work has been done to determine the mechanism of action of LH on the bovine luteal cell. This work is summarized in Fig. 1.

The initial requirement for LH-stimulated progesterone synthesis is the binding of the LH molecule to a receptor in the plasma membrane. By definition, a receptor must exhibit a high degree of specificity and affinity for the respective hormone (Ryan and Lee, 1976). The first evidence for a plasma membrane LH receptor was found using autoradiographic techniques in rat luteal tissue (Rajaniemi and Vanha-Perttula, 1972). Binding of radiolabelled LH and hCG is also used to quantitate gonadotropin receptors. The bovine luteal cell contains  $5 \times 10^4$  binding sites for hCG (Papalouannou and Gospodarowicz, 1975). This number is probably variable however, since Diekman et al. (1978a) have reported that the number of LH receptors in the ovine CL increases from day 2 to day 10 of the estrous cycle and declines around day 16. These workers suggested that the number of LH receptors may be the major factor in the regulation of  $P_4$  secretion, but this conclusion is questionable since only 0.6% of the total receptors were occupied by LH on day 10 of the cycle.

The LH receptor is glycoprotein in nature (Azhar and Menon, 1981b) and has a molecular weight of approximately 240,000 (Rao et al., 1981). The interaction of the gonadotropin with the receptor appears to be at least partially regulated by the phospholipids in the plasma membrane, since binding capacity is inhibited after treatment of membranes with phospholipase A or C (Azhar and Menon, 1976; Azhar et al., 1976b).

The binding of LH to the plasma membrane receptor initiates a

series of cellular responses which ultimately lead to increased  $P_4$  production. In isolated luteal cells, unoccupied LH receptors are randomly distributed over the plasma membrane. LH binding induces clustering of the occupied receptors into small aggregates or patches (Luborsky and Behrman, 1979). This reorganization of receptors may facilitate two subsequent events: 1) coupling to the enzyme adenylate cyclase and 2) internalization of the hormone-receptor complex.

### Adenylate Cyclase

Occupied gonadotropin receptors can interact with adenylate cyclase, a plasma membrane-associated enzyme which converts MgATP to cAMP. The adenylate cyclase enzyme consists of two components: 1) a catalytic unit which converts MgATP to cAMP and 2) a guanine nucleotide-binding regulatory component (N) (reviewed by Rodbell, 1980; Ryan, 1982; and Birnbaumer, 1982). N is formed of two subunits,  $n_\alpha$  and  $n_\beta$ . The occupied gonadotropin receptor couples with  $n_\beta$  and causes a shift in the  $K_m$  of N for Mg, from 10mM to less than 0.5mM, which is the physiological concentration of Mg. Once Mg binds to N (probably to  $n_\beta$ ), there is a dissociation of  $n_\alpha$  from  $n_\beta$ , allowing  $n_\alpha$  to interact with and activate the catalytic unit. There is also a requirement for the nucleotide GTP, which, when associated with  $n_\alpha$ , allows it to bind to the catalytic unit. This active form of adenylate cyclase is then able to convert MgATP to cAMP. Therefore, hormones act primarily by reducing the Mg required for GTP-dependent activation of adenylate cyclase (Birnbaumer, 1982).

The coupling of the hormone-receptor complex to adenylate cyclase may depend on the lateral diffusion of these proteins throughout the plasma membrane. This movement of membrane proteins is likely to be influenced by the degree of membrane fluidity. Hanski et al. (1979) and

Rimon et al. (1980) have provided evidence to suggest that the turkey erythrocyte  $\beta$ -adrenergic receptor can only activate the cyclase in fluid areas of the membrane, and that receptor-mediated cyclase activation is a diffusion controlled process. The degree of membrane fluidity also has a direct effect on the cyclase catalytic unit, independent of the effect on the receptor-cyclase interaction (Hanski et al., 1979). Furthermore, the fluidity of the lipid bilayer can affect the number of receptors available for hormone binding. Mouse liver membranes treated with prostacyclin exhibit both a 17% decrease in membrane microviscosity and a 40-50% increase in the number of receptors for prolactin (Dave and Knazek, 1980). Therefore, membrane fluidity may affect the gonadotropin receptor, the activity of adenylate cyclase, and the interaction between the two.

Recently, data have been presented indicating the converse might also be true, i.e. the gonadotropin-receptor complex may alter the fluidity of the plasma membrane. Milvae et al. (1982) observed that inhibitors of phospholipid methylation were able to block LH-stimulated  $P_4$  production by the bovine CL. This is consistent with the theory that ligand binding to the membrane receptor initiates a series of trans-methylation reactions within the plasma membrane resulting in the conversion of phosphatidylethanolamine to phosphatidylcholine. The increased PC/PE ratio causes the membrane to be more fluid (reviewed by Axelrod and Hirata, 1982). Therefore, hormone binding may bring about an increase in membrane fluidity which would, in turn, increase lateral mobility and coupling to the adenylate cyclase molecule.

#### Desensitization

Interestingly, the binding of LH or hCG to its receptor may also

induce a transient refractoriness or desensitization of the cell to further stimulation. Conn et al. (1978) injected pseudopregnant rats with  $^{125}\text{I}$ -hCG and reported extensive internalization of the hormone-receptor complex by 7 hours post-injection. Further evidence that the gonadotropin may enter the target cell has been presented for the ovine CL (Ahmed et al., 1981), dissociated rat (Luborsky and Behrman, 1979) and monkey (Gulyas et al., 1981) luteal cells, and cultured rat granulosa cells (Amsterdam et al., 1979). The internalization of the hormone-receptor complex leads to a down-regulation of LH receptors, rendering the cell refractory to the gonadotropin.

This is not the single mechanism for desensitization, however, since desensitization occurs much more rapidly than down-regulation (Ryan, 1982). The initial phase of desensitization appears to be due to the inability of the hormone-receptor complex to couple with the adenylate cyclase molecule (Hall and Behrman, 1981; Birnbaumer, 1982), and may be brought about by the clustering and decreased mobility of receptors (Amsterdam et al., 1980) or by a phosphorylation of either the receptor or the regulatory subunit of the cyclase (Birnbaumer, 1982).

The functional significance of the desensitized state is not clear, but it may serve as a homeostatic mechanism to protect the cells from variations in hormone concentrations. The internalization of the hormone-receptor complex is important as a means for the peptide hormone to regulate its own receptors (Catt et al., 1979), and may be involved in the chronic rather than acute, effects of the gonadotropin (Conn et al., 1978). Indeed, internalized hCG remains biologically active for at least 6 hours (Zimniski et al., 1982), and the presence of hCG receptors in the nuclear membranes, lysosomes, rough endoplasmic reticulum, and golgi

of the bovine CL (Rao et al., 1981) raises the possibility that the peptide hormone might induce specific effects at the subcellular level.

#### Cyclic AMP and cAMP-Dependent Protein Kinase

As previously stated, activation of adenylate cyclase results in the formation of cAMP, the proposed second messenger of LH action in luteal tissue. Addition of cAMP to bovine luteal tissue slices incubated *in vitro* caused an increase in  $P_4$  synthesis (Marsh and Savard, 1966), and treatment of luteal slices with LH resulted in a rapid accumulation of cAMP which preceded the rise in  $P_4$  (Marsh et al., 1966). It was subsequently demonstrated that the LH-stimulated increase in endogenous cAMP was due to stimulation of adenylate cyclase, rather than to an inhibition of phosphodiesterase, the enzyme responsible for the degradation of cAMP (Marsh, 1970).

Ling and Marsh (1977) reported that it was possible to stimulate  $P_4$  production with very low levels of LH (10 ng/ml) without an increased accumulation of cAMP. However, there was a concomitant increase in cAMP-dependent protein kinase activity, suggesting that cAMP had been produced, but the levels were below the sensitivity of the assay. More recently, elevated levels of cAMP have been measured at all doses of LH able to stimulate steroidogenesis in both rat (Sala et al., 1979) and bovine (Ling et al., 1980) luteal tissue, providing further evidence that cAMP is an intracellular mediator of LH action.

The many diverse effects of cAMP are exerted through the activation of a cytoplasmic protein kinase. This protein kinase has been purified and characterized from the bovine corpus luteum (Menon, 1973), and its activation has been shown to play a role in gonadotropin-stimulated steroidogenesis (Vaitukaitis et al., 1975; Azhar et al., 1976a).



Cyclic AMP-dependent protein kinase is composed of a regulatory and a catalytic subunit (Walsh and Ashby, 1973). When cAMP binds to the regulatory subunit it leads to the dissociation and activation of the catalytic subunit. The catalytic subunit is then capable of phosphorylating a number of intracellular proteins, and a correlation between protein phosphorylation and  $P_4$  synthesis has been observed in bovine luteal cells (Darbon et al., 1981). It has been hypothesized that LH-stimulated cAMP is compartmentalized and activates protein kinase in specific subcellular compartments, leading to the phosphorylation of specific, rather than all, substrates of protein kinase (Hayes and Brunton, 1982).

In 1976 Marsh listed five possible functions of cAMP-activated protein kinase: 1) an increase in a cofactor such as NADPH, via the phosphorylase system, 2) an increase in the concentration of cholesterol, 3) an increase in the movement of cholesterol into the mitochondria, 4) a direct action on the mitochondrial side-chain cleavage system, and/or 5) an enhancement of the efflux of pregnenolone out of the mitochondria. Although evidence was presented to support each of these proposals, it was clear that no single action of protein kinase could be chosen. In 1982 Ryan stated that the substrate for cAMP-dependent protein kinase in the ovary is still unknown. Possible cellular sites of action include the nucleus (Jungmann et al., 1974), the mitochondria (Downing and Dimino, 1979), and microsomal proteins (Azhar and Menon, 1975). It is generally accepted that LH-stimulated steroidogenesis requires RNA and protein synthesis (Marsh, 1976), and this is probably under the influence of protein kinase. Furthermore, Simpson et al. (1978) have shown that in the adrenal, the protein that is required for

steroidogenesis acts intramitochondrially. This protein serves to increase the binding of cholesterol to cytochrome P-450, the enzyme responsible for the conversion of cholesterol to pregnenolone.

#### Cholesterol Side-Chain Cleavage

There are three potential sources of cholesterol which can be used for steroidogenesis; it can be synthesized de novo from acetate (Hellig and Savard, 1965), it can enter the cells from the circulation (Shima et al., 1968; Bolte et al., 1974; Christie et al., 1979), or it can be derived from stored cholesteryl ester pools (Coutts and Stansfield, 1968).

The availability of the cholesterol stored in the ester form is influenced by protein kinase. Behrman and Armstrong (1969) observed that LH treatment caused the depletion of sterol esters and suggested that this was due to a stimulation of cholesteryl esterase activity. Trzeciak and Boyd (1973) and Beckett and Boyd (1977) were the first to report that adrenocortical cholesteryl esterase is activated by cAMP-dependent protein kinase. Cholesteryl esterase exists in an inactive, dephosphorylated form. Phosphorylation and activation is catalyzed by protein kinase, resulting in hydrolysis of cholesteryl esters and an increase in free cholesterol available for steroidogenesis. In the bovine CL, Bisgaler et al., (1979) also observed the activation of sterol ester hydrolase by  $N^{6,2}$ -dibutyryl cyclic AMP and demonstrated that the level of activation was significantly decreased in the presence of a protein kinase inhibitor.

Cholesterol in its free form must be transported to the mitochondria for metabolism to steroid hormones. In the adrenal, cholesterol is transported through the cytoplasm by a sterol carrier protein (SCP<sub>2</sub>)

(Chanderbhan et al., 1982). Once in the mitochondria the cholesterol can be converted to pregnenolone by a reaction known as side chain cleavage. Side chain cleavage of cholesterol is the rate-determining step in steroidogenesis, and is also considered to be the major site of action of LH. This intramitochondrial event has recently been reviewed by Strauss et al. (1981) and Kimura (1981). The conversion of cholesterol to pregnenolone and a six-carbon fragment, isocaproate, involves an enzyme system which consists of three components: 1) luteodoxin reductase, an FAD-containing flavoprotein, 2) cytochrome P-450<sub>scc</sub>, the terminal oxygenase, and 3) luteodoxin, which serves to shuttle an electron between the reductase and P-450. The side chain cleavage reaction involves two sequential hydroxylations at C<sub>22</sub> and C<sub>20</sub> of cholesterol and the actual cleavage of the cholesterol side chain between C<sub>20</sub> and C<sub>22</sub>. Each step in the reaction mechanism requires molecular oxygen and NADPH; therefore, the conversion of one mole of cholesterol to one mole of pregnenolone requires three moles of oxygen and three moles of NADPH. The slowest part of the reaction is the association of cholesterol with the P-450 molecule, and it is thought that LH may act by increasing the availability and/or binding of substrate by cytochrome P-450.

The cytochrome P-450 molecule is located within the inner layer of the inner mitochondrial membrane. Kimura (1981) proposed that the cholesterol which is also located in this portion of the membrane is readily available for binding to P-450. Cholesterol located in the outer mitochondrial membrane, or the outer layer of the inner membrane is not able to interact with the side chain cleavage enzyme. Seybert et al. (1979) described "metabolizable" and "non-metabolizable" pools of cholesterol, and suggested that ACTH acted by stimulating the rate of

delivery of cholesterol to the metabolizable pool. Kimura (1981) states that this involves a flip-flop of cholesterol across the membrane. Although this is a likely site of action for LH, the exact mechanism is not known. Currently there are two theories, involving the action of either a labile protein or specific phospholipids.

Protein synthesis is necessary for LH-stimulated steroidogenesis, and a labile protein generated on cytoplasmic ribosomes is thought to be translocated to the mitochondria (reviewed by Strauss et al., 1981). As previously stated, Simpson et al. (1978) have proposed that the labile protein increases the binding of cholesterol to cytochrome P-450<sub>scc</sub> in adrenal mitochondria. Toaff et al. (1979) have also reported that in luteal mitochondria, movement of cholesterol to the side chain cleavage system is the primary factor determining steroidogenic capacity, and this process is inhibited by cycloheximide, an inhibitor of protein synthesis.

Cycloheximide is also able to inhibit synthesis of polyphosphoinositide in adrenal tissue, and ACTH stimulates the synthesis of this phospholipid (Farese and Sabir, 1980). It has been suggested that polyphosphoinositide is the cytosolic factor that enhances the side chain cleavage reaction, and synthesis of this phospholipid requires the ACTH-stimulated labile protein (Farese and Sabir, 1980; Farese et al., 1981).

Davis et al. (1981) reported that LH stimulated  $^{32}\text{PO}_4$  incorporation into certain phospholipids in bovine luteal cells, implying a role for phospholipids similar to that in the adrenal, but Scott et al. (1968) and Strauss and Flickinger (1977) were unable to detect an effect of LH on phospholipid metabolism. More recently however, Tanaka and Strauss

(1982) were able to stimulate luteal cholesterol side chain cleavage with the phospholipid cardiolipin, even though hCG caused no major changes in phospholipids. These authors speculated that phospholipid changes may occur in specialized regions of the mitochondria and were not detected by their methods.

Once cholesterol has been converted to pregnenolone, the pregnenolone passes out of the mitochondria to the smooth endoplasmic reticulum, where the enzymes  $\Delta^5-3\beta$ -hydroxysteroid dehydrogenase and  $\Delta^5-\Delta^4$  isomerase are located (Tamaoki, 1973). Together, these enzymes bring about the transformation of pregnenolone to progesterone. The major products of the bovine CL are pregnenolone, progesterone, and its metabolite  $20\beta$ -dihydroprogesterone (Mason et al., 1962). While Savard and Telegdy (1965) stated that the bovine CL did not have the enzymes necessary to synthesize androgens from progestins, Shemesh et al. (1975) reported in vitro synthesis of testosterone by luteal slices that was further stimulated by LH, arachidonic acid, and  $\text{PGF}_{2\alpha}$ . Although it is still questionable whether or not the bovine CL does synthesize testosterone, most evidence indicates that this tissue is not able to aromatize androgens to estrogens (Savard and Telegdy, 1965; Henderson and Moon, 1979; Koos and Hansel, 1981; and Ursely et al., 1981).

#### Progesterone Secretion

Once synthesized, the progesterone must be secreted from the luteal cell into the bloodstream. Little is known about the mechanism involved in the transport or release of the steroid from the luteal cell (Cavazos, 1972). It has been generally assumed that steroids leave cells by simple diffusion (Vogt, 1943) as soon as they are synthesized (Enders, 1973). In 1974, Rubin et al. reported that the perfused cat

adrenal gland secreted a protein concomitantly with corticosteroids, and suggested that corticosteroid release did not occur by simple diffusion, but might occur by exocytosis.

The idea that a steroid might be secreted by exocytosis would require that the steroid be packaged in some form, with release of a number of steroid molecules from a single site. In an ultrastructural study of the cyclic ovine CL, Gemmell et al. (1974) described the presence of small, densely staining granules which increased and decreased in number concurrently with the known pattern of  $P_4$  secretion of the gland. These workers concluded that  $P_4$  secretion in the corpus luteum occurs by exocytosis of these secretory granules. The secretory granules which are also present in the bovine CL (Quirk et al., 1979; Parry et al., 1980), are thought to contain  $P_4$  bound to a carrier protein (Gemmell and Stacy, 1979a; Sawyer et al., 1979), which is expelled from the cell when the membrane surrounding the granule fuses with and becomes incorporated into the plasma membrane (Gemmell and Stacy, 1979b).

Leymarie and Gueriguan (1970) first reported the presence of  $P_4$  binding activity in the soluble fraction of the bovine CL. A high-affinity binding protein for  $P_4$  was later described (Willcox and Thorburn, 1981), which was released along with  $P_4$  during *in vitro* incubation (Willcox and Alison, 1982). These results tend to support the contention that intracellular  $P_4$  is bound to a protein and released via a secretory granule.

Cytochemical investigations aimed at characterizing the nature of the secretory granules have had conflicting results. Quirk et al. (1979), utilizing sucrose density gradient centrifugation of bovine luteal tissue homogenates, have found electron dense granules in frac-

tions which contained a high enrichment of progesterone. These authors support the hypothesis that progesterone is sequestered within the secretory granules, and further state that a protein is probably present within the granules to render them electron dense. Sernia et al. (1982) attempted to extend these observations using ovine luteal tissue and examined the binding of  $P_4$  to protein within the granules. However, they were unable to find any progesterone-binding activity in the fraction containing the granules, or in the medium of corpus luteum slices incubated in vitro. It was admitted that there is no conclusive evidence that  $P_4$  is transported by these granules in a protein-bound form.

In trying to elucidate the mechanism of steroid secretion, it became clear from the work of Vinson et al. (1979) that two steroids (corticosterone and 18-hydroxydeoxycorticosterone from the adrenal cortex) can be released by the same cell independently. Corticosterone appears to be secreted by simple diffusion while secretion of 18-OH-DOC involves a more complex process (Sibley et al., 1980; Sibley et al., 1981). Since the output of the two steroids varied independently, it was suggested that the terms "synthesis" and "secretion" were not necessarily synonymous, although these two terms are generally used interchangeably when referring to steroid production. This concept has been supported by the work of Condon and Pate (1981) who showed that synthesis and secretion of  $P_4$  from the bovine CL could be altered independently in vitro.

Further support for the idea that secretion of a steroid does not always occur at the same rate as its synthesis has come from work involved with the cytoskeleton of steroidogenic cells. Gemmell and Stacy (1977) injected ewes with colchicine, a drug which specifically

disrupts the microtubular system, and found a buildup of  $P_4$  in luteal tissue, but a decrease in plasma  $P_4$ . This was supported using an in vitro system of ovine luteal slices (Sawyer et al., 1979). Also, Bassett and Pollard (1980) suggested that microtubules are involved in the process of steroid secretion in the rat adrenal in vivo.

In contrast, Azhar and Menon (1981a) and Gwynne and Condon (1982) were not able to demonstrate an effect of colchicine on rat or bovine luteal cells in vitro. However, the microfilament modifier, cytochalasin B, was able to decrease LH-stimulated  $P_4$  levels in ovine, rat, and bovine luteal cells (Silavin et al., 1980; Azhar and Menon, 1981a; and Gwynne and Condon, 1982), but this was due to an effect on synthesis, not secretion, of the steroid (Gwynne and Condon, 1982). This more recent evidence seems to indicate that neither microtubules nor microfilaments are involved in the secretory process of  $P_4$ , but microfilaments appear to be involved in the actual synthesis of  $P_4$ . It is possible that the role of microfilaments may be to transport cholesterol to the mitochondria (Crivello and Jefcoate, 1980), or to regulate cholesterol synthesis by modifying the activity of HMG-CoA reductase (Volpe and Obert, 1981).

Although the mechanism responsible for the intracellular transport of  $P_4$  to the plasma membrane remains to be elucidated, it is well-established that once released, the steroid travels through the blood bound to a protein. Cook et al. (1977) have shown that bovine plasma contains at least two progesterone-binding proteins, albumin and corticosteroid-binding globulin. Eik-Nes (1975) and Westphal (1980) have suggested that the presence of serum steroid-binding proteins increases steroid secretion, and this has been supported by in vitro work in the



testis (Ewing et al., 1976) and CL (Condon and Pate, 1981).

### Cholesterol Metabolism

Cholesterol is a necessary substrate for  $P_4$  synthesis in the CL (Bloch, 1945; Wilks et al., 1970). As previously mentioned, the steroidogenic cells have three possible sources from which to obtain free cholesterol: newly synthesized, stored and circulating cholesterol. Although the CL of the cow possesses all of the enzymes required for the total synthesis of cholesterol (Savard, 1973), Armstrong et al. (1970) have reported that LH is able to increase luteal  $P_4$  production in vitro even in the presence of a cholesterol synthesis inhibitor. LH also decreased  $^{14}C$ -acetate incorporation into sterol, indicating that cholesterol synthesized de novo was probably not the major substrate for hormone synthesis in the acutely stimulated luteal cell.

The corpus luteum is also able to accumulate large amounts of cholesterol in storage granules, which have been described by Armstrong and Flint (1973). These membrane-bound granules contain cholesteryl ester, cholesterol, protein and phospholipid, and shrink upon trophic stimulation of the tissue, as the cholesterol is utilized for steroid formation. Cholesterol is esterified by acyl coenzyme A:Cholesterol acyltransferase, which is located in the microsomal membrane, and the cholesteryl ester is cleaved by the cytosolic enzyme cholesteryl ester hydrolase. The rate of esterification and ester hydrolysis is reflected in the activities of ACAT and CEH, which may be regulated by gonadotropins, substrate supply, steroidogenic end-products, and stage of the estrous cycle (Caffrey et al., 1979; Tavani et al., 1982; reviewed by Strauss et al., 1981). Although the bovine CL has been shown to have cholesteryl ester stores and an active CEH (Coutts and Stansfield, 1968;

Bisgaler et al., 1979), the importance of this enzyme is not clear, since the CL of the cow stores much larger quantities of free cholesterol than cholesteryl ester (Hafs and Armstrong, 1968).

The role of exogenous cholesterol as a precursor for steroidogenesis has been extensively studied in recent years. When exogenous cholesterol is lowered luteal steroidogenesis is greatly decreased despite increased de novo sterol synthesis and reduced sterol ester storage (Christie et al., 1979; Schuler et al., 1979). Strauss et al. (1981) have calculated that maximal rates of de novo synthesis can provide only 10% of the cholesterol required to meet ovarian demands.

Although rat luteal cells (Azhar and Menon, 1981c) and cultured rat granulosa cells (Rosenblum et al., 1981) are dependent upon exogenous cholesterol for maximal steroidogenesis, free cholesterol is a poor substrate for these cells. The majority of the cholesterol in the blood is carried in the form of a lipoprotein molecule, and this lipoprotein-carried cholesterol may serve as a substrate for steroidogenesis. It is likely that steroidogenic cells utilize low density lipoprotein in a manner similar to that advanced by Brown and Goldstein (1976) for cultured human fibroblasts. Briefly, the LDL molecule binds to a specific cell surface receptor, clusters into specialized regions of the cell known as coated pits, and becomes internalized into the cell by a process known as receptor-mediated endocytosis. The LDL is transported to the lysosome where it is degraded, releasing free amino acids and cholesteryl ester, which is further hydrolyzed to yield free cholesterol. This cholesterol is then available for membrane synthesis, steroidogenesis or storage. The majority of evidence linking lipoprotein uptake to steroidogenesis has been obtained from studies with the adrenal cortex

and has been reviewed by Brown et al. (1979). Indeed, of the many fresh tissues examined by Kovanen et al. (1979), the adrenal cortex exhibits the largest number of high affinity binding sites for LDL, while the corpus luteum ranks second.

Since the pioneering work of Brown and Goldstein (1976), a wealth of evidence has been presented supporting the utilization of lipoprotein-derived cholesterol for steroidogenesis in many species, and has been extensively reviewed by Gwynne and Strauss (1982). Investigations involving the cow have so far relied solely on in vitro systems, but cultures of bovine granulosa cells (Savion et al., 1982) and luteal cells (Pate and Condon, 1982) exhibit increased  $P_4$  production in the presence of LDL. In the rat, lipoprotein-supplied cholesterol is the major substrate for the manufacture of steroid hormones in the adrenal, ovary and testis in vivo, and this species preferentially utilizes HDL-cholesterol rather than LDL-cholesterol (Andersen and Dietschy, 1978).

The mechanism of HDL uptake is incompletely understood. The HDL binding sites are distinct from the LDL receptor but are not so precisely characterized. Rat luteal cells appear to use HDL-cholesterol without prior or simultaneous degradation of the apolipoproteins (Schuler et al., 1981a), though rat granulosa cells in culture are capable of degrading rat and human HDL (Schreiber et al., 1982). HDL increases  $P_4$  production by bovine granulosa cells and luteal cells in culture (Savion et al., 1982; Pate and Condon, 1982), presumably by providing cholesterol to be used as substrate for steroidogenesis. Further study is required to determine how the HDL-cholesterol is released into the cell.

The utilization of various cholesterol sources for steroidogenesis and cellular maintenance results in a dynamic equilibrium of choles-

sterol metabolism in luteal cells. Cholesterol synthesis, esterification and hydrolysis are adjusted in accordance with a balance between the metabolic needs of the cell and the supply of exogenous cholesterol. Regulation is at least partially exerted through modulation of ACAT and HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis, while CEH and the cholesterol side-chain cleavage enzyme do not appear to be regulated by cholesterol balance (Schuler et al., 1981b). These latter enzymes are, however, under the influence of trophic hormone stimulation. When lipoprotein-carried cholesterol is sufficient to meet the needs of the cell the free cholesterol will increase the activity of ACAT, resulting in a rise in sterol ester synthesis. At the same time HMG-CoA reductase, and hence de novo sterol synthesis, are suppressed. If steroidogenesis increases or lipoprotein uptake is limited, HMG-CoA reductase is stimulated and ACAT is suppressed; cellular cholesterol synthesis is thus enhanced, and the sterol ester pool is mobilized (Schuler et al., 1981b).

The cholesterol balance of the cell is most likely altered by the secretory state of the cell (Strauss et al., 1981), and is therefore influenced, either directly or indirectly, by the trophic hormone. Indirectly, the gonadotropin may alter enzyme function by increasing intracellular steroid levels and lowering free cholesterol. It is also likely that cholesterol uptake is directly affected by the gonadotropin, since bovine ovaries perfused with LH accumulate more  $^3\text{H}$ -cholesterol than non-stimulated ovaries (Bartosik and Romanoff, 1969), and ACTH stimulates the uptake of plasma cholesterol by the rat adrenal (Dexter et al., 1970). The transfer of HDL-cholesterol into rat adrenal cells incubated in vitro is increased in the presence of ACTH (Gwynne et al.,

1976), and Kovanen et al. (1980) were able to increase the numbers of adrenal LDL receptors 2- to 5- fold in mice or rats by administration of ACTH. It would obviously be physically advantageous if LH increased luteal uptake of lipoprotein-cholesterol concurrently with the stimulation of steroidogenesis.

### Luteolysis

The transient nature of the corpus luteum is probably its most unique feature. "Its ephemerality is its most distinguishing and important characteristic...and if there is a basic, common system of regulation of all mammalian CL, the clue to its nature must be in what causes ephemerality" (Rothchild, 1981).

Normal luteal regression involves both autophagic and heterophagic processes, as indicated by the presence of lysosomes and activated macrophages associated with the morphological destruction of the CL (McClellan et al., 1977; Paavola, 1979). However, this is a relatively slow process since functional luteolysis preceeds, and is much more rapid than structural luteolysis (Umo, 1975). Functional luteolysis is marked by a precipitous decline in plasma  $P_4$  and a loss of the ability of the CL to respond to trophic stimulation. Although the CL had been under gonadotrophic influence for maximal progesterone synthesis, a mechanism other than simply the withdrawal of pituitary support appears to exist for the initiation of luteolysis (McCracken et al., 1973).

In 1956, Wiltbank and Casida reported that hysterectomy prolonged the life span of the CL in sheep and cows. Since that time it has become clear that the presence of the uterus is necessary for normal luteal regression. More specifically, experiments with unilaterally hysterectomized ewes and heifers during normal and artificially induced

luteolysis revealed that the retained uterine horn must be ipsilateral to the ovary bearing the CL in order for regression to occur. This suggested a local effect of the uterus on the ovary (Hansel et al., 1973).

### Prostaglandin $E_{2\alpha}$

In 1966, Babcock first suggested that a prostaglandin might be the substance released from the uterus which causes luteolysis. Pharriss and Wyngarden (1969) injected  $PGF_{2\alpha}$  into rats and noted a drop of serum  $P_4$  and a shortening of pseudopregnancy. Since  $PGF_{2\alpha}$  was an abundant uterine prostaglandin they postulated that it might be the uterine luteolysin. Administration of  $PGF_{2\alpha}$  was later found to induce luteal regression in both laboratory and domestic animals (reviewed by Ellinwood et al., 1978; Inskeep, 1973). McCracken et al. (1972) measured a rise in uterine secretion of  $PGF_{2\alpha}$  in cyclic ewes which correlated with the drop in  $P_4$ . Also, infusion of  $PGF_{2\alpha}$  into the ovarian blood supply caused luteolysis, and it was concluded that  $PGF_{2\alpha}$  was the uterine luteolytic hormone in the ewe. Further evidence to support this conclusion in sheep has been reviewed by Goding (1974).  $PGF_{2\alpha}$  has also been implicated as the uterine luteolysin in the cow; levels of this prostaglandin rise around the time of luteolysis (Nancarrow et al., 1973), and indomethacin injected into the uterine horn adjacent to the CL prevented normal luteal regression in ewes and helpers (Lewis and Warren, 1977).

The bovine CL does have receptors for  $PGF_{2\alpha}$  (Powell et al., 1975), and the binding affinity of the receptor for  $PGF_{2\alpha}$  increases 203-fold from day 13 to day 20 of the estrous cycle (Rao et al., 1979).  $PGF_{2\alpha}$  binding is low in the early luteal phase in the cow, but receptors are

present (Rao et al., 1979), while in the rat the affinity and capacity of  $\text{PGF}_{2\alpha}$  receptors do not change between days 4 and 10 (Wright et al., 1980). This is interesting because the CL is refractory to the effects of  $\text{PGF}_{2\alpha}$  on days 1-4 of the estrous cycle in the cow (Inskeep, 1973; Henricks et al., 1974). Rao et al. (1979) and Wright et al. (1980) do not attribute this refractoriness to a lack of PG receptors. Wise et al. (1982) have suggested that  $\text{PGF}_{2\alpha}$  may not reach the CL early in the cycle because blood flow is shunted toward the uterus and away from the ovary during this time

#### Counter-Current Theory

If  $\text{PGF}_{2\alpha}$  is the uterine luteolysin, and if it acts directly on the corpus luteum, then a unique transport mechanism must exist to transport the molecule from the uterus to the ovary. If prostaglandin leaves the uterus and travels through the systemic circulation, most of the biological activity will be lost upon passage through the lungs. In sheep and cows the ovarian artery is highly convoluted and lies on the surface of the utero-ovarian vein before entering the hilus of the ovary. This anatomical feature of the utero-ovarian vasculature prompted the idea that the luteolytic hormone might diffuse out of the utero-ovarian vein and into the ovarian artery by a counter-current mechanism and travel directly to the ovary. This process would account for the necessity of the uterus to be ipsilateral to the CL-bearing ovary. The utero-ovarian vasculature of many species and the evidence supporting the counter-current theory have been extensively reviewed by Ginther (1974) and McCracken et al. (1973).

The first evidence to support a counter-current theory of prostaglandin transport came from the work of McCracken et al. (1972). These

workers infused  $^3\text{H-PGF}_{2\alpha}$  into the uterine veins of ewes and recovered the labelled compound in much greater quantities from ovarian arterial blood than from peripheral samples. A number of similar experiments have supported this work, and Alwachi et al. (1981) have pointed out that prostaglandin may pass to the ovarian artery via the oviducal vein as well as from the utero-ovarian vein. While it may seem questionable that a prostaglandin molecule can diffuse through the arterial wall, recent evidence has shown that larger steroid molecules may also be capable of counter-current transfer. Walsh et al. (1979) and Elner-Jensen and McCracken (1981) have reported diffusion of  $\text{P}_4$  from the ovarian vein into the ovarian artery in sheep, and the transfer of testosterone within the ovarian vascular pedicle may occur in the cow (Kotwica et al., 1982). This mechanism for providing the ovary with high concentrations of ovarian-secreted steroids may serve as a means of local regulation of ovarian function.

Although the counter-current theory is generally accepted in the sheep, it has not been firmly established that this mechanism exists in the cow. Shemesh and Hansel (1975a) observed that concentrations of  $\text{PGF}_{2\alpha}$  rose in the uterine vein on days 15-17 of the estrous cycle, but they could not measure an increase in the ovarian artery. Milvae and Hansel (1980a) obtained similar results after injections of oxytocin to stimulate uterine release of  $\text{PGF}_{2\alpha}$ . Milvae and Hansel (1983) later postulated that perhaps a metabolite of  $\text{PGF}_{2\alpha}$  was transferred to the ovarian artery and was not detected by their assay for  $\text{PGF}_{2\alpha}$ . They did find one metabolite of  $\text{PGF}_{2\alpha}$ , 13,14-dihydro- $\text{PGF}_{2\alpha}$ , to be luteolytic in heifers.

#### Possible Mechanisms of Action of $\text{PGF}_{2\alpha}$



While it is recognized that  $\text{PGF}_{2\alpha}$  is luteolytic in many species, the manner in which it brings about regression of the CL is unknown.  $\text{PGF}_{2\alpha}$  is a potent vasoconstrictor, and therefore might act on the vasculature to restrict the blood supply to the ovary. Niswender et al. (1976) have reviewed the relationship between blood supply and ovarian function, and noted that during regression the blood flow to the ovary was decreased. This also occurred during  $\text{PGF}_{2\alpha}$ -induced luteolysis, and it was suggested that the decreased blood flow might limit the availability of LH to the luteal cells. While Niswender (1981) has reported a decrease in ovarian blood flow within 2 hours after  $\text{PGF}_{2\alpha}$  injection in sheep, Wehrenberg et al. (1979) could not measure any decrease in blood flow following  $\text{PGF}_{2\alpha}$  administration in guinea pigs. Since there are functional arterio-venous anastomoses in sheep ovaries (Mattner et al., 1981), it is possible that blood could be diverted away from the CL without a change in overall blood supply to the ovary. However, in  $\text{PGF}_{2\alpha}$ -injected pseudopregnant rats, Pang and Behrman (1979) did not detect a change in blood flow to the CL before the drop in plasma  $\text{P}_4$ . The differences reported here could be due to species differences, or to the techniques employed to measure changes in blood supply. Obviously, more work must be done to determine if  $\text{PGF}_{2\alpha}$  causes luteolysis by vasoconstriction.

Since the CL contains receptors for  $\text{PGF}_{2\alpha}$  it would seem reasonable that this prostaglandin could exert a direct effect on the CL itself. During luteolysis in the rat, the ratio of  $\text{P}_4$  to its metabolite  $20\alpha$ -dihydroprogesterone is greatly decreased (Wiest and Kidwell, 1969; Aakvaag and Torjesen, 1981). It has been suggested that  $\text{PGF}_{2\alpha}$  may lower circulating  $\text{P}_4$  by accelerating its breakdown (Lau et al., 1979), and

this is supported by the work of Jones and Hsueh (1981) who measured a  $\text{PGF}_{2\alpha}$ -induced increase in  $20\alpha$ -hydroxysteroid dehydrogenase activity in cultured rat granulosa cells.  $\text{PGF}_{2\alpha}$  can also decrease other luteal enzymes, such as  $3\beta$ -hydroxysteroid dehydrogenase (Dwyer and Church, 1979), cholesterol esterase and cholesterol synthetase (Behrman et al., 1971), which would interrupt the normal cholesterol balance and steroidogenic pathway of the cell.

In 1976, Grinwich et al. showed that  $\text{PGF}_{2\alpha}$ -induced luteolysis was accompanied by a decrease in the number of LH receptors in the rat corpus luteum. This observation was later confirmed in the ovine (Diekman et al., 1978b) and bovine (Spicer et al., 1981) CL, but in these latter cases the decrease in LH receptors occurred at least 12 hours after the fall in plasma  $\text{P}_4$ . It would seem, therefore, that the loss in gonadotropin receptors would be a chronic effect of  $\text{PGF}_{2\alpha}$ , rather than the initial cause of the  $\text{P}_4$  decline.

Probably the most intriguing aspect of  $\text{PGF}_{2\alpha}$  action is the disparate results obtained during in vitro incubation of luteal tissue with this hormone. In 1 to 4 hour incubations of bovine luteal tissue slices (Speroff and Ramwell, 1970; Hansel et al., 1973) or dispersed luteal cells (Hixon and Hansel, 1979),  $\text{PGF}_{2\alpha}$  stimulated progesterone production above control levels. This effect of the prostaglandin was difficult to reconcile with its known luteolytic action in vivo. However, O'Grady et al. (1972) and Hall and Robinson (1979) reported that  $\text{PGF}_{2\alpha}$  inhibited basal progesterone production by incubated luteal tissue from the rabbit and rat, respectively. In further studies, it was found that  $\text{PGF}_{2\alpha}$  had no effect on basal levels of  $\text{P}_4$ , but was able to suppress LH-stimulated  $\text{P}_4$  in tissue slices from the rat and the ewe (Evrard et al., 1978) and

in dispersed cells from the rat (Wright et al., 1980). These findings are similar to those of Thomas et al. (1978) who utilized a 2 hour culture of rat luteal cells. In this system,  $\text{PGF}_{2\alpha}$  stimulated basal  $\text{P}_4$  production in a dose-dependent manner, but LH-stimulated steroidogenesis was completely inhibited. However, if dbcAMP was added to the culture medium, the effects of the prostaglandin were overcome, prompting the authors to conclude that the mechanism of action of  $\text{PGF}_{2\alpha}$  was to block the LH-dependent formation of cAMP.

Using a different approach, Jordan (1981) injected rats with  $\text{PGF}_{2\alpha}$ , then removed the luteal tissue and examined in vitro steroidogenesis. Luteal cells from  $\text{PGF}_{2\alpha}$ -treated rats were less responsive to both LH and dbcAMP in vitro, and it was concluded that the major site of action of  $\text{PGF}_{2\alpha}$  was at a point distal to the accumulation of cAMP. The reason for the differences between these last two studies is not known, but it may be that  $\text{PGF}_{2\alpha}$  has divergent effects on the luteal cell in vivo and in vitro. Khan et al. (1979) and Khan and Rosberg (1979) were able to inhibit LH-stimulated adenylate cyclase activity in rat corpora lutea by prior administration of  $\text{PGF}_{2\alpha}$  to the animals, but it was not determined if there was a lesion in the steroidogenic pathway beyond cAMP production. In contrast to these studies, Weston and Hixon (1980) reported that while  $\text{PGF}_{2\alpha}$  administered to cows caused a decline in plasma and luteal  $\text{P}_4$  concentrations, the tissue remained responsive to LH during subsequent in vitro incubation. Due to paradoxical effects of  $\text{PGF}_{2\alpha}$  in vivo and in vitro, and the variability in the response to the prostaglandin in vitro, the mechanism of action of  $\text{PGF}_{2\alpha}$  has thus far remained elusive.

A novel concept concerning corpus luteum regression has recently

been proposed to involve changes in membrane fluidity. Using wide angle x-ray diffraction, Buhr et al. (1979) and Carlson et al. (1982) have shown that rat and bovine luteal cell membranes undergo a change from a liquid phase to a gel phase during spontaneous and  $\text{PGF}_{2\alpha}$ -induced luteolysis. The increase in membrane viscosity is thought to be partly due to an increased concentration of sphingomyelin in membranes of the regressing CL (Goodsaid-Zalduondo et al., 1982), and it is suggested that this is an effect of exposure of the CL to  $\text{PGF}_{2\alpha}$ . If  $\text{PGF}_{2\alpha}$  is able to cause the luteal cell membrane to become less fluid, this would obviously influence the steroidogenic potential of the cell. Membrane gonadotropin receptors and adenylate cyclase molecules would be immobilized, severely reducing the opportunity for coupling and rendering the cell unresponsive to gonadotropin stimulation. This model for  $\text{PGF}_{2\alpha}$  action appears promising, but it must be determined if the membrane phase changes occur as a rapid response to  $\text{PGF}_{2\alpha}$ , or if the alterations in membrane fluidity are a consequence of previously initiated cell death.

#### Oxytocin and Luteolysis

In 1959, Armstrong and Hansel noted that daily injections of oxytocin during the first week of the bovine estrous cycle resulted in premature regression of the CL and a shortened cycle. Staples and Hansel (1961) reported that oxytocin caused a depletion of luteal progesterone. This luteolytic effect of oxytocin requires the presence of the uterus, and Ginther et al. (1967) showed that in unilaterally hysterectomized helpers, oxytocin induces luteal regression only if the retained horn is ipsilateral to the ovary bearing the CL, suggesting a local utero-ovarian mechanism for oxytocin action.

In 1976, Roberts and McCracken reported that oxytocin infused into the uterus of the cycling ewe stimulated uterine production of  $\text{PGF}_{2\alpha}$  on day 3 and day 14 of the estrous cycle. In vitro incubation of ovine endometrial tissue with oxytocin results in increased synthesis of  $\text{PGF}_{2\alpha}$  (Roberts et al., 1976), and McCracken (1980) has postulated that the luteolytic effects of oxytocin could be mediated through  $\text{PGF}_{2\alpha}$ .

Recently, a high concentration of oxytocin was found to be present in the ovine CL (Wathes and Swann, 1982), and these authors suggested that it was of luteal origin. This idea was supported by the work of Flint and Sheldrick (1982), who measured oxytocin in ovarian venous and arterial blood. The concentration of oxytocin was much greater in ovarian venous blood and was increased by injection of a  $\text{PGF}_{2\alpha}$  analogue. Jugular venous concentrations of oxytocin parallel those of  $\text{P}_4$  during the ovine estrous cycle, suggesting that this protein hormone is being released from the CL concurrently with  $\text{P}_4$  (Flint and Sheldrick, 1983). Although this work seems to support the idea that oxytocin is stored in and secreted by the CL, it is not known if the intraluteal oxytocin is a result of uptake and storage from the blood or de novo synthesis of oxytocin. The presence of oxytocin in the CL raises the question of whether this peptide hormone is involved in normal luteal function and regulation of the estrous cycle. Flint and Sheldrick (1983) also measured increased oxytocin concentrations coinciding with the surges of  $\text{PGF}_{2\alpha}$  around the time of luteolysis. Since oxytocin stimulates  $\text{PGF}_{2\alpha}$  release from the endometrium, and it appears that  $\text{PGF}_{2\alpha}$  increases ovarian secretion of oxytocin, it is proposed that at the time of luteal regression, a positive feedback loop is established between the oxytocin from the ovary and the  $\text{PGF}_{2\alpha}$  from the uterus to ensure the demise of the

CL. The idea of a positive feedback is not entirely new, since Rothchild (1981) has indicated that many aspects of luteal function, both luteotropic and luteolytic, may rely on positive feedback mechanisms.

#### Intraluteal Prostaglandins

The bovine CL is able to synthesize PGF, and this synthesis is stimulated by LH (Shemesh and Hansel, 1975b; Hixon and Hansel, 1979). Rat granulosa cells *in vitro* can also synthesize prostaglandins, and LH increases prostaglandin synthetase activity (Clark et al., 1978; Koos and Clark, 1982). Although it may seem contradictory that a tissue would synthesize the very compound that will eventually cause its destruction, this fact may provide an important insight into understanding the transient nature of the CL. Rothchild (1981) has postulated that intraluteal prostaglandin synthesis is the luteolytic signal in the corpora lutea of all species. According to his theory, the ability of the CL to make prostaglandin is inversely related to its ability to make progesterone. Rothchild believes that  $P_4$  is the primary stimulus of its own secretion, and that  $P_4$  suppresses luteal prostaglandin production at the same time that it increases the CL's potential ability to synthesize prostaglandins. When  $P_4$  reaches a critical level, prostaglandin synthesis is switched on and is also self-stimulating, resulting in further increases in intraluteal  $PGF_{2\alpha}$  concentrations and regression of the corpus luteum. Therefore, the CL is regulated by positive feedback mechanisms, and the natural instability of positive feedbacks leads to the demise of the tissue.

"...the cause of its ephemerality, which is to say, the basic and common elements of regulation that determine the CL's ability to secrete

progesterone, and that brings this secretion and with it the life of the CL as a whole to an end, must lie within the CL itself, in all species" (Rothchild, 1981).

## MATERIALS AND METHODS

### Tissue Collection and Culture

Corpora lutea were removed per vaginum from regularly cycling, dry dairy cows of various breeds on days 8-12 of the estrous cycle and placed into Ham's F-12 culture medium (Microbiological Associates) containing 24mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (Hepes, Calbiochem-Behring Corp.) (pH 7.4) at 4 °C. The tissue was weighed, sliced, minced, and transferred to a spinner flask containing Ham's F-12-Hepes medium with 0.5% bovine serum albumin (BSA, fraction V, Sigma). The minced tissue was magnetically stirred for 15 min at 35°C. The medium was then decanted and replaced with fresh medium containing collagenase (2000 U/g tissue, Type I, Worthington). The tissue was aspirated into a 10 ml pipette every 10 min to facilitate dissociation. After 45 min the dissociation medium was decanted and centrifuged (100xg, 10 min). Fresh medium with collagenase was added to the remaining tissue for an additional 45 min of dissociation. Cells from both dissociations were pelleted, the supernatant discarded, and the cell pellets were washed 4 times with fresh medium. Cells were pooled, counted in a hemocytometer and viability was determined by the trypan blue exclusion method (Tennant, 1964).

Approximately  $1 \times 10^6$  cells were seeded into 25 cm<sup>2</sup> polystyrene tissue culture flasks (Corning) in a final volume of 4 ml of Ham's F-12-Hepes culture medium containing 100 U/ml penicillin, 100 ug/ml streptomycin, 2.5 ug/ml fungizone (Grand Island Biological Company) and supplemented with either 10% dialyzed calf serum (GIBCO) or with insulin (2



ug/ml, Sigma), hydrocortisone (40 ng/ml, Collaborative Research) and transferrin (5 ug/ml, Collaborative Research). In the prostaglandin experiments, medium was supplemented with insulin (5 ug/ml), transferrin (5 ug/ml) and selenium (5 ng/ml) (Collaborative Research). For cultures that were not to receive serum, the culture flasks were pretreated with Ham's F-12 culture medium containing 10% serum for 1 hour at 37°C to coat the bottom of the flask with the attachment proteins contained in the serum. This serum-containing medium was then removed and the flask was rinsed 1 time with a double volume of Ham's F-12.

Cultures were incubated at 37°C. Culture medium was removed after 24 hr, replaced with fresh medium, and subsequently changed every 48 hr, for 11 days. Unless otherwise indicated, all treatments were added at the beginning of the culture and replaced with each medium change. All culture medium was stored at -20°C until assayed for P<sub>4</sub>. The number of animals used in each experiment is shown in the tables and in the legends to the figures. All treatments were performed in duplicate for each animal.

Cells were quantitated throughout the culture period using an ocular micrometer square to count numbers of cells growing in the culture flask. Each flask was counted in 5 locations, a minimum of 3 times during the culture period. Steroidogenic activity was then normalized per  $5 \times 10^5$  viable cells growing in monolayer culture.

#### Lipoprotein Isolation and Quantitative Assays

Lipoproteins were prepared from normal bovine serum obtained from blood collected from dry dairy heifers. Low-density lipoprotein (LDL) (density 1.006-1.063 g/ml) and high-density lipoprotein (HDL) (1.063-1.210 g/ml) were prepared by differential ultracentrifugation using KBr

for density adjustments (Havel et al., 1955). The lipoprotein fractions were dialyzed against 0.15 M NaCl containing 0.34 mM EDTA at pH 7.4, and sterilized by passage through a 0.22  $\mu$ m Millipore filter. Lipoproteins were stored at 4°C and used within 4 weeks of preparation.

Cholesterol in the lipoprotein fractions was determined by the method of Wybenga et al. (1970). Progesterone in the spent culture medium was quantitated by radioimmunoassay of unextracted samples with anti-progesterone-11-BSA serum (GDN-337) donated by Dr. Gordon Niswender. This antiserum does not cross-react significantly with any abundant steroid in our samples (Gibori et al., 1977). [1,2-<sup>3</sup>H] Progesterone was obtained from New England Nuclear. The intraassay coefficient of variation was 10.7%, the interassay coefficient of variation was 16.8%. The limit of sensitivity was 0.1 ng/ml. All standards and samples were assayed in duplicate. Details of the progesterone RIA procedure are given in the appendix. Prostaglandins were determined by radioimmunoassay (Lewis et al., 1978) in the laboratory of Dr. Keith Inskeep at West Virginia University.

#### Acetate Incorporation Study

Freshly dissociated luteal cells were cultured in serum-free medium as described, with 6 control flasks and 6 cultures treated with LH (10 ng/ml). On day 1 (24 hr after plating), the medium was replaced with fresh medium containing <sup>14</sup>C-acetate (0.25  $\mu$ Ci/ml, New England Nuclear). Two control and 2 LH-treated cultures also received LDL (50  $\mu$ g lipoprotein-cholesterol/ml), and another 2 control and 2 LH-treated cultures received HDL (50  $\mu$ g cholesterol/ml). Cells were incubated for 24 hours at 37°C. This procedure was repeated on day 5 and day 11 with an additional 12 flasks on each day.

At the end of the 24-hr exposure to  $^{14}\text{C}$ -acetate, 1 ml of medium from each flask was removed and frozen for assay of  $\text{P}_4$ . The remaining medium was extracted 2x with 5 ml anhydrous ether. The cell monolayers were rinsed with phosphate buffered saline, drained, and extracted with 5 ml hexane:isopropanol (3:2 v:v) for at least 5 min. The hexane:isopropanol and ether extracts were combined, dried under air at  $37^\circ\text{C}$ , and stored at  $-20^\circ\text{C}$ .

For thin layer chromatography, samples were reconstituted in benzene:ethanol (9:1) and applied to Silica Gel GF<sub>254</sub> TLC plates (Fisher Scientific). One-dimensional chromatography was performed in benzene:methylene chloride (1:1) followed by hexane:ethyl acetate (5:2).  $\text{P}_4$  and cholesterol spots were eluted with methanol (3 mls, 2x).

Samples were counted in a Beckman LS7000 liquid scintillation counter and radioactivity was expressed as disintegrations per minute or specific activity.

#### Treatment Preparation

All treatment dilutions were prepared in phosphate-buffered saline immediately prior to use, except for forskolin, which was made up in ethanol, and kept on ice until added to the cultures. All treatments were added in aliquots of 100  $\mu\text{l}$  or less. The treatments used, their supplier, and concentrations employed were:

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TREATMENT	CONCENTRATIONS
<hr/>	
1. Bovine LH (NIAMMD-bLH-4)	10,100, and 1000 ng/ml
2. Dibutyryl cAMP (Sigma)	1.02 mM
3. Bovine Prolactin (NIAMMD-bPRL-6)	10,20,50,100 and 1000 ng/ml
4. Indomethacin (Merck, Sharp & Dohme)	10 ug/ml
5. PGF <sub>2α</sub> (Sigma)	10,100 and 1000 ng/ml
6. PGE <sub>1</sub> (Sigma)	10 and 1000 ng/ml
7. Cholera Toxin (Calbiochem)	1 nM
8. Forskolin (Calbiochem)	10 nM
9. hCG (Calbiochem)	10 ng/ml

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### Statistical Analysis

Differences in progesterone levels between treatments were analyzed by the paired t-test or by analysis of variance and Newman-Keuls multiple range test (Zar, 1974).

## RESULTS

### Serum vs. Serum-Free Medium

The effect of LH on progesterone production by luteal cells cultured in medium containing 10% dialyzed calf serum is summarized in Table 1. LH (10 and 100 ng/ml) did not increase  $P_4$  production above control values except on day 7 with the higher level of LH.

In contrast, cells cultured in serum-free medium were responsive to LH. As shown in Fig. 2, all levels of LH (10, 100 and 1000 ng/ml) were able to significantly increase progesterone production on day 1 ( $P<0.001$ ) and day 3 ( $P<0.01$ ) of culture. Although the absolute levels of  $P_4$  declined throughout the culture period, this did not reflect the ability of the cells to respond to gonadotropin. In Fig. 3,  $P_4$  production is expressed as a percentage above control values to indicate the responsiveness of the cells to LH (10 ng/ml) throughout the 11 days of culture. In the presence of serum (dashed line), LH was unable to increase progesterone production above control levels at any time during the culture period, except for the slight rise on day 7. However, LH was able to cause a significant ( $P<0.01$ ) increase in steroidogenesis in the serum-free cultures, and these cells exhibited a very consistent pattern of responsiveness to LH. The cultures were highly responsive to LH on day 1, the response then declined somewhat on days 3 and 5, but returned by days 7-11 ( $P<0.01$ ).

Since the serum-free medium was supplemented with insulin, transferrin and hydrocortisone, it is possible that the LH response was due to their presence rather than to the absence of serum. To examine this

possibility, luteal cells were cultured in medium containing 10% dialyzed calf serum but also supplemented with insulin, transferrin and hydrocortisone (Table 2). The addition of these three components to the serum-containing medium did not permit the cells to respond to LH to any greater extent than in serum-containing medium alone.

Fig. 4 illustrates the effect of dbcAMP on progesterone production in cultured bovine luteal cells. In both the presence and absence of serum, dbcAMP (solid line) was able to stimulate progesterone synthesis. However, only in serum-free medium was LH, when added in combination with dbcAMP (dashed line), able to cause a further increase in  $P_4$  above that produced by the dbcAMP alone.

The morphology of bovine luteal cells in culture is shown in Figs. 5 and 6. In serum-containing medium (Fig. 5), the densely packed cells each possess a nucleus with prominent nucleoli, numerous cytoplasmic lipid droplets, and cytoplasmic extensions. In serum-free medium (Fig. 6), nucleoli are also noticeable, cytoplasmic processes and points of attachment are quite obvious, but the lipid droplets are much less numerous.

#### Effects of Prolactin, $PGE_1$ and Cell Density

To examine the possibility of long term effects of prolactin on the bovine CL, cells were cultured for 11 days in serum-free medium in the presence of various concentrations of bovine prolactin (0, 10, 50 and 1000 ng/ml). For comparison to LH, duplicate cultures received 10 ng/ml LH or prolactin (50 ng/ml) + LH. The results of this experiment are depicted in Fig. 7, and  $P_4$  is expressed as a % above control values. While there appears to be a slight stimulation of  $P_4$  production induced by prolactin, this increase is not significant ( $P > 0.05$ ) at any levels of

prolactin tested. The combination of prolactin plus LH was able to increase  $P_4$  ( $P < 0.001$ ) above controls, but this stimulation was not different from that produced by LH alone.

Prostaglandins of the E series generally stimulate adenylate cyclase in ovarian preparations, so the influence of  $PGE_1$  on  $P_4$  production in cultured luteal cells was investigated. The addition of  $PGE_1$  (10 and 1000 ng/ml) to serum-free cultures resulted in an elevation of  $P_4$  above controls ( $P < 0.01$ , Fig. 8). The pattern of responsiveness to  $PGE_1$  was similar to that seen with LH (Fig. 3), i.e., the cells were very responsive on day 1 of culture, less responsive in the middle of the culture, and showed increased responsiveness on days 7-11.

To determine the optimal cell density for cellular response to LH, luteal cells were plated at densities ranging from  $2.5 \times 10^5$  to  $2 \times 10^6$  cells/flask, in 4 ml of serum-free medium. Plating efficiency was slightly less than 50% in these experiments, and progesterone production was calculated as ng/62,500, 125,000, 250,000 or 500,000 cells. In Fig. 9,  $P_4$  production is again expressed as a percent above control levels to indicate the responsiveness of the cells to LH during the 11-day culture period. The cells plated at the lower densities tended to be the most responsive during the first 24 hr of culture (125 and 130% above controls), but the responsiveness is then low throughout the remainder of the culture period. The cultures containing approximately 250,000 cells (plated at  $1 \times 10^6$  cells/flask) responded to LH on day 1 (117%); this response then declined but returned on days 9 and 11 to 73% and 61% respectively. This degree of responsiveness at the end of the time period examined was greater than that found with the higher density cultures (plated at  $2 \times 10^6$  cells/flask). For this reason, in all subse-

quent experiments  $1 \times 10^6$  cells were plated per culture flask. Because only two animals were used for this study and the variance between the two was high, there was no statistical significance between treatments.

#### Lipoprotein Experiments

Although removing the serum from the culture medium resulted in a more responsive system, the progesterone output by the luteal cells was somewhat decreased. This could be due to the absence of the cholesterol contained in the serum, which might serve as substrate for  $P_4$  synthesis. To investigate this possibility, freshly isolated bovine lipoproteins (LDL and HDL) were added to the cultured luteal cells (Fig. 10). When the cells were exposed to the lipoproteins during the first 24 hr of culture (day 1), LDL did not increase progesterone synthesis, while HDL produced a 1.5-fold increase over control levels. The addition of LH increased progesterone production in both lipoprotein-free and lipoprotein-treated cultures, indicating that neither LDL nor HDL inhibited the LH response, as had the serum. This same pattern is apparent on day 3, after exposure to the lipoproteins for an additional 48 hr.

In order to deplete endogenous cholesterol stores, the remaining cultures were allowed to grow in serum-free medium for 3 days before lipoprotein addition. At this time, either LDL or HDL was added to the cultures and incubated for 48 hr (day 5). In this case both LDL and HDL greatly increased progesterone levels without affecting the LH response. Continual exposure to the lipoproteins through days 7 and 9 of culture produced similar increases above non-lipoprotein-treated controls.

Lipoproteins may increase  $P_4$  production by providing cholesterol as a substrate for steroidogenesis. Therefore, it is likely that de novo cholesterol synthesis and subsequent conversion of this cholesterol to



$P_4$  would be lowered in the presence of lipoproteins. This was examined in the cultured luteal cells using  $^{14}\text{C}$ -labelled acetate incorporation into sterols and steroids as an indicator of de novo synthesis. Figs. 11-15 illustrate the results of this study. Due to the low number of animals and variability of radioactively labelled product, these results were not statistically significant, but some general trends are apparent. On days 2 and 6 of culture (Figs. 11 and 12, respectively), the presence of LDL in the culture medium resulted in a decrease in the incorporation of  $^{14}\text{C}$ -acetate into cholesterol. This trend was also true for HDL in two of the three animals. On day 11 of culture (Fig. 13) both LDL and HDL were able to reduce de novo cholesterol synthesis in cells from all three animals, and HDL appeared to be more effective than LDL.

Both LDL and HDL also lower the specific activity of the progesterone produced by the cultured cells in the presence of  $^{14}\text{C}$ -acetate on days 2, 6 and 11 (Fig. 14). When LH is combined with the lipoproteins there is still a decrease in the incorporation of  $^{14}\text{C}$ -acetate into  $P_4$  on all three days, and LH alone lessens the specific activity of the  $P_4$  fraction on days 2 and 11 (Fig. 15).

#### Effects of Indomethacin and Prostaglandin

To study the luteolytic action of  $\text{PGF}_{2\alpha}$  in the bovine CL, initial experiments were undertaken to determine the effects of  $\text{PGF}_{2\alpha}$  and a prostaglandin synthesis inhibitor, indomethacin, on  $P_4$  production by luteal cells in culture for 11 days. Fig. 16 illustrates the synthesis of 6-keto- $\text{PGF}_{1\alpha}$  by cultured luteal cells on days 1, 5, and 11. This prostaglandin is a stable metabolite of prostacyclin and is used as an indicator of prostacyclin synthesis. On all three days LH caused a

slight but nonsignificant rise in 6-keto-PGF<sub>1α</sub>. The addition of indomethacin, either alone or in combination with LH, completely inhibited synthesis of 6-keto-PGF<sub>1α</sub> on days 1 and 5. Although not significant on day 11, a similar trend was seen.

Synthesis of PGF<sub>2α</sub> and PGE<sub>2</sub> by luteal cells from one animal is shown in Table 3. LH appeared to increase PGF<sub>2α</sub> synthesis on days 1, 5 and 11, and PGE<sub>2</sub> synthesis on days 1 and 11. It should be noted, however, that the standard deviation between duplicate LH-treated cultures is very high in the PGE<sub>2</sub> assay on day 5. Indomethacin completely abolished PGF<sub>2α</sub> synthesis, and this effect could not be overcome by the addition of LH. Indomethacin lowered, but did not seem to cause a total inhibition of PGE<sub>2</sub> synthesis. On days 5 and 11, PGE<sub>2</sub> values represent one sample and therefore have no standard deviation.

While indomethacin blocks luteal prostaglandin synthesis, it is also capable of promoting luteal progesterone formation (Table 4). On days 1 and 3, indomethacin has no effect on P<sub>4</sub> production, but continued treatment results in a large increase in P<sub>4</sub> (days 5-11). This is evident in the presence or absence of LH.

Table 5 lists P<sub>4</sub> values obtained by treating the cultured cells with PGF<sub>2α</sub>. P<sub>4</sub> was elevated in medium collected on day 1 of culture after exposure to either 10 or 1000 ng/ml of PGF<sub>2α</sub>. Further exposure to 10 ng/ml PGF<sub>2α</sub> had no effect on steroidogenesis, but 1000 ng/ml PGF<sub>2α</sub> was again stimulatory at the end of the culture period (days 9 and 11).

#### Mechanism of Action of PGF<sub>2α</sub>

In order to probe the PGF<sub>2α</sub> mechanism of action in isolated luteal cells, cultures were treated with PGF<sub>2α</sub> alone and in combination with various steroidogenic stimulants. Fig. 17 illustrates the effects of LH

and  $\text{PGF}_{2\alpha}$  on days 1, 3 and 5 of culture. On day 1, LH and  $\text{PGF}_{2\alpha}$  + LH increased  $\text{P}_4$  above control levels. There was no difference between LH- and  $\text{PGF}_{2\alpha}$  + LH-treated cultures. Neither  $\text{PGF}_{2\alpha}$  nor LH had any effect on  $\text{P}_4$  on day 3. However, on day 5, LH alone increased  $\text{P}_4$  production while cultures treated with LH +  $\text{PGF}_{2\alpha}$  (10 or 1000 ng/ml) were not different from controls. Similar results were obtained on days 7-11 (Fig. 18); although neither level of  $\text{PGF}_{2\alpha}$  had any effect on basal  $\text{P}_4$  production, both prostaglandin doses completely inhibited LH-stimulated steroidogenesis. Since there was no difference between results obtained with 10 or 1000 ng/ml  $\text{PGF}_{2\alpha}$  in these experiments, only the lower dose of  $\text{PGF}_{2\alpha}$  will be shown in the remaining figures.

Fig. 19 depicts the effects of different treatment agonists, alone and combined with  $\text{PGF}_{2\alpha}$ , on the first day of culture. Again,  $\text{PGF}_{2\alpha}$  had no effect on basal or LH-stimulated  $\text{P}_4$  production. Cholera toxin, a direct stimulator of the regulatory subunit of adenylate cyclase, and forskolin, which also directly stimulates adenylate cyclase activity, caused an increase in  $\text{P}_4$  on day 1, and this increase was not altered by the simultaneous addition of  $\text{PGF}_{2\alpha}$ . Neither indomethacin nor indomethacin +  $\text{PGF}_{2\alpha}$  had any effect on steroidogenesis on day 1.

After 3 days of culture, CT, FKN and INDO stimulated  $\text{P}_4$  production above controls, and in all cases this stimulation was blocked by concurrent treatment with  $\text{PGF}_{2\alpha}$  (Fig. 20). LH was not stimulatory on day 3.

Further analyses on days 5, 7 and 9 of culture are shown in Figs. 21, 22 and 23, respectively. Throughout this interval of the culture,  $\text{P}_4$  was increased by each of the treatment agonists (LH, CT, FKN and INDO). In all cases, although  $\text{PGF}_{2\alpha}$  had no effect on basal steroidogenesis, it completely inhibited the agonist-induced increases in  $\text{P}_4$ .

On day 11, an identical trend can be seen (Fig. 24), but in this case only FKN produced a significant stimulation.

In addition to these experiments, similar studies were undertaken using dbcAMP and hCG as the treatment agonists. Results are shown for days 1 (Fig. 25), 5 (Fig. 26) and 9 (Fig. 27) of culture. On day 1, both dbcAMP and hCG tended to increase  $P_4$  and this effect was not prevented by the addition of  $PGF_{2\alpha}$ . On days 5 and 9, hCG stimulated steroidogenesis, and  $PGF_{2\alpha}$  blocked this hCG effect. Although dbcAMP produced only a slight increase in  $P_4$  on days 5 and 9, it appeared that  $PGF_{2\alpha}$  inhibited any stimulatory action of dbcAMP.

#### Morphology of Hormone-Treated Luteal Cells

Control cultures of luteal cells (no treatment additives) grown in serum-free medium exhibit an elongated appearance and tend to arrange themselves in parallel fashion (Fig. 28). Some lipid droplets are present and are generally dispersed throughout the cytoplasm, often in line with stress fibers. In contrast, LH-stimulated cells assume an epithelial-like shape and lipid droplets are usually located around the nucleus (Fig. 29). There are very few cytoplasmic processes, and little, if any, cell-cell contact is seen.

Fig. 30 shows cells that have been treated with  $PGF_{2\alpha}$ . In general, these cells appear similar to LH-stimulated cells; they are epithelioid in shape, contain perinuclear lipid droplets, and are usually separated from one another by a distinct cell-free space. When  $PGF_{2\alpha}$  and LH are combined (Fig. 31), the result is a culture that looks like the individually treated cultures. Cells are either epithelioid or round with perinuclear lipid. Each cell is separated from all others by a distinct space, with no cell-cell contacts or obvious cytoplasmic extensions.

TABLE 1  
EFFECT OF LH ON PROGESTERONE PRODUCTION BY LUTEAL CELLS  
CULTURED IN SERUM-CONTAINING MEDIUM

----- Progesterone (ug/500,000 cells) <sup>a</sup> -----			
Days in Culture	Control	LH (10 ng/ml)	LH (100 ng/ml)
-----			
1	21.77 ± 2.05	22.87 ± 3.47	
	19.23 ± 2.41		22.97 ± 2.11
-----			
3	11.86 ± 3.26	15.21 ± 4.90	
	5.69 ± 0.76		6.69 ± 1.29
-----			
5	4.06 ± 1.12	4.63 ± 1.69	
	2.25 ± 0.50		2.82 ± 0.65
-----			
7	2.60 ± 1.42	3.57 ± 1.89	
	0.96 ± 0.54		1.36 ± 0.65 <sup>b</sup>
-----			
9	2.95 ± 1.64	2.11 ± 1.23	
	0.75 ± 0.53		1.03 ± 0.94
-----			
11	1.64 ± 0.87	2.03 ± 1.24	
	0.49 ± 0.27		0.60 ± 0.39
-----			

<sup>a</sup>MEAN ± SEM; n=5

<sup>b</sup>P<0.05

TABLE 2  
EFFECT OF LH ON P<sub>4</sub> PRODUCTION BY  
LUTEAL CELLS IN MEDIUM CONTAINING SERUM,  
INSULIN, TRANSFERRIN AND HYDROCORTISONE

-----		
	Progesterone (ug/500,000 cells) <sup>a</sup>	
Days In	-----	
Culture	Control	LH (10 ng/ml)
-----		
1	10.27 ± 2.98	15.26 ± 4.27
3	9.25 ± 1.11	12.22 ± 0.19
5	4.16 ± 0.56	5.02 ± 0.70 <sup>b</sup>
7	3.15 ± 0.80	3.60 ± 1.45
9	2.32 ± 0.82	2.22 ± 0.81
11	1.55 ± 0.86	1.56 ± 0.81
-----		

<sup>a</sup>MEAN ± SEM; n=2

<sup>b</sup>P<0.05

TABLE 3

PGF<sub>2α</sub> AND PGE<sub>2</sub> SYNTHESIS BY CULTURED BOVINE LUTEAL CELLS

Treatment	Day 1	Day 5	Day 11
PGF <sub>2α</sub> (pg/500,000 cells) <sup>a</sup>			
Control	8031 ± 864	3375 ± 920	2862 ± 744
LH (10 ng/ml)	9157 ± 938	3474 ± 1292	4399 ± 1357
Indo (10 ug/ml)	24 ± 0	24 ± 0	24 ± 0
Indo + LH	24 ± 0	24 ± 0	24 ± 0
PGE <sub>2</sub> (pg/500,000 cells) <sup>a</sup>			
Control	1614 ± 252	1150 ± 774	2 ± 0
LH (10 ng/ml)	4136 ± 3498	674 ± 282	664 ± 13
Indo (10 ug/ml)	863 ± 243	197	30
Indo + LH	677 ± 389	262	118

<sup>a</sup>MEAN ± SD; n=1

Indo = Indomethacin

TABLE 4  
EFFECT OF INDOMETHACIN ON BASAL AND LH-STIMULATED  
PROGESTERONE SYNTHESIS

Treatment	n	Progesterone (ng/500,000 cells) <sup>a</sup>		
		Day 1	Day 3	Day 5
Control	6	2938 ± 212	1529 ± 229	617 ± 69
LH (10 ng/ml)		4211 ± 435 <sup>d</sup>	1492 ± 333	615 ± 39
Indo (10 ug/ml)		2469 ± 311	1599 ± 205	784 ± 82 <sup>d</sup>
Indo + LH		4708 ± 420 <sup>d</sup>	1869 ± 347 <sup>c</sup>	798 ± 84 <sup>d</sup>
		Day 7	Day 9	Day 11
Control	5	426 ± 97	267 ± 57	207 ± 45
LH (10 ng/ml)		451 ± 79	359 ± 70 <sup>b</sup>	274 ± 71 <sup>b</sup>
Indo (10ug/ml)		641 ± 127 <sup>c</sup>	666 ± 164 <sup>c</sup>	525 ± 93 <sup>d</sup>
Indo + LH		843 ± 103 <sup>c</sup>	781 ± 201 <sup>c</sup>	565 ± 97 <sup>d</sup>

<sup>a</sup>MEAN ± SEM

<sup>b, c, d</sup> different from control, P<0.05, P<0.01, P<0.001, respectively



TABLE 5  
INFLUENCE OF PGF<sub>2α</sub> ON BASAL PROGESTERONE SYNTHESIS

Treatment	Progesterone (ng/500,000 cells) <sup>a</sup>		
	Day 1	Day 3	Day 5
Control	2439 ± 323	1572 ± 177	917 ± 78
PGF <sub>2α</sub> (10 ng/ml)	3143 ± 471 <sup>c</sup>	1635 ± 245	852 ± 57
PGF <sub>2α</sub> (1000 ng/ml)	3439 ± 661 <sup>b</sup>	1533 ± 181	877 ± 933
	Day 7	Day 9	Day 11
Control	529 ± 73	351 ± 49	279 ± 48
PGF <sub>2α</sub> (10 ng/ml)	499 ± 55	366 ± 58	272 ± 50
PGF <sub>2α</sub> (1000 ng/ml)	604 ± 122	472 ± 96 <sup>b</sup>	425 ± 113 <sup>b</sup>

<sup>a</sup>MEAN ± SEM; n=8

<sup>b</sup>different from control P<0.01

<sup>c</sup>different from control P<0.001

Figure 1. Summary diagram of luteotropism.

Membrane

AC = adenylate cyclase  
 C = catalytic subunit  
 N = regulatory subunit  
 PE = phosphatidylethanolamine  
 PME = phosphatidyl-N-monomethylethanolamine  
 PC = phosphatidylcholine  
 P-lipids = phospholipids  
 R = receptor

Cytoplasm

PK = protein kinase  
 R = regulatory subunit  
 C = catalytic subunit  
 AA's = amino acids  
 ACAT = acyl coenzyme A:cholesterol acyltransferase  
 CEH = cholesterol ester hydrolase  
 FFA = free fatty acid

Mitochondria

P-450 = cytochrome P-450<sub>scc</sub>

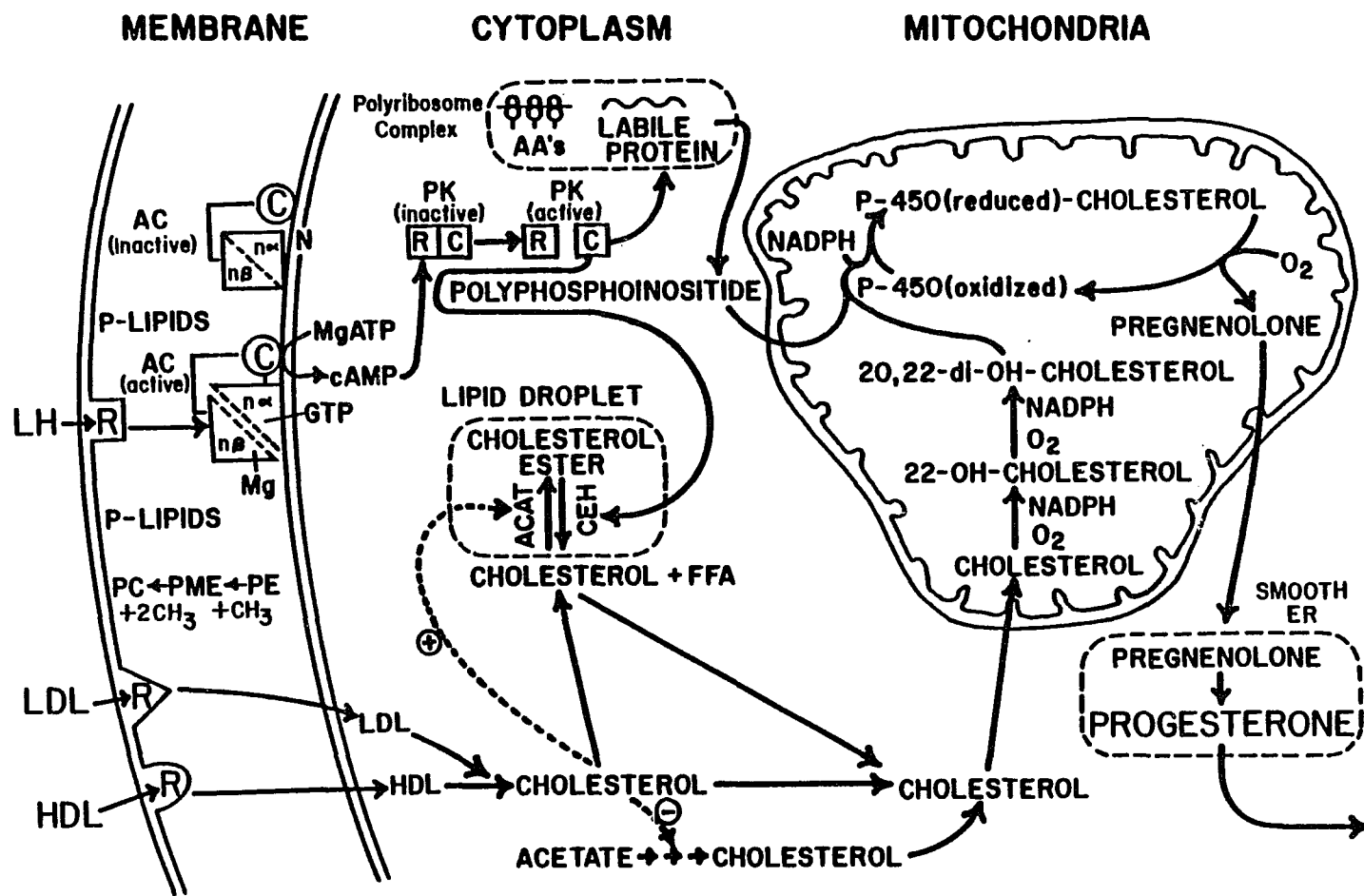


FIGURE 1

Figure 2. Effect of LH on progesterone production by luteal cells cultured in serum-free medium. Luteal cells were cultured in Ham's F-12 medium supplemented with insulin (2 ug/ml), hydrocortisone (40 ng/ml) and transferrin (5 ug/ml) in the absence or presence of LH (10, 100 and 1000 ng/ml). Spent culture medium was removed on day 1 (24 h after the beginning of culture). The cultures then received fresh medium and LH for an additional 48 h, i.e. day 3. Mean  $\pm$  SEM, n = 8. Values statistically different from the control group are indicated: \*\*P<0.01, \*\*\*P<0.001.

FIGURE 2

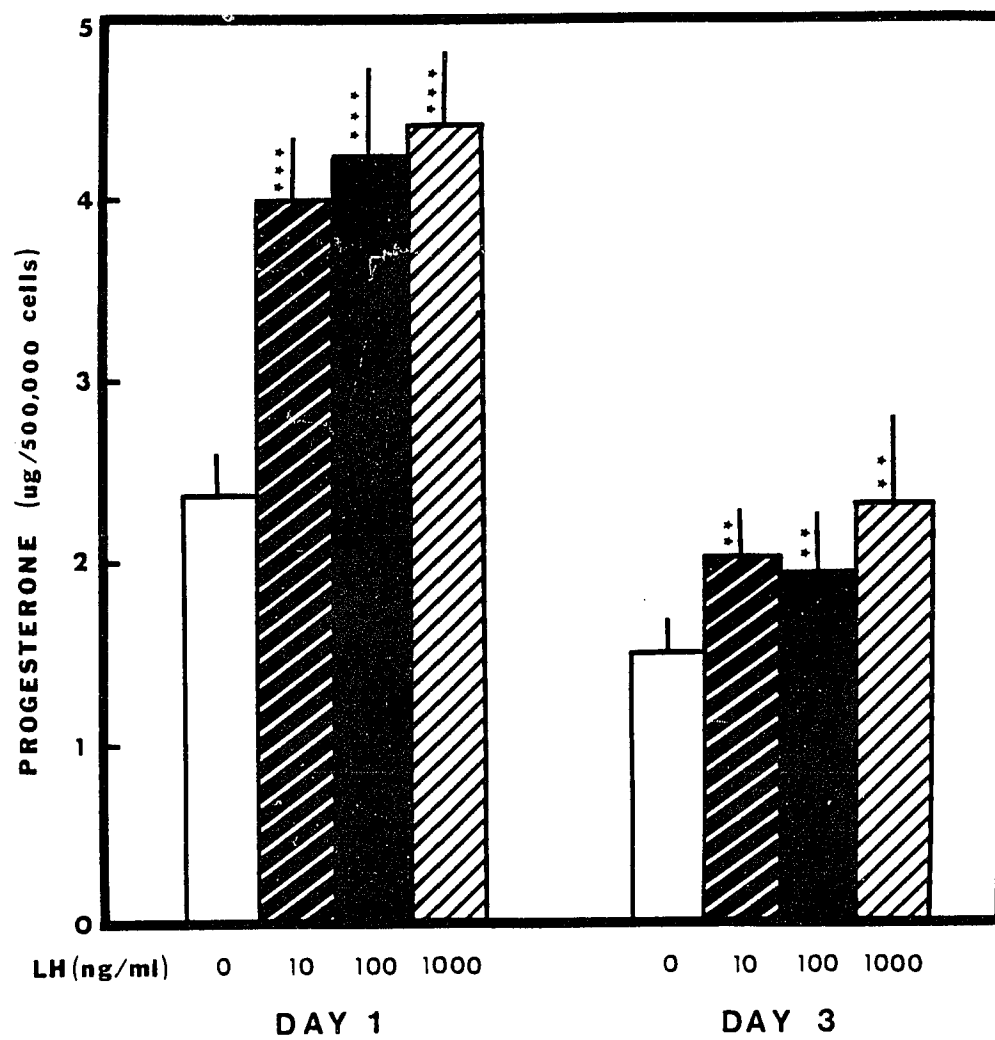


Figure 3. Responsiveness of luteal cells to LH (10 ng/ml) in serum-free and serum-containing medium. LH was added at the beginning of the culture and replaced with each medium change. Control values for progesterone on days 1, 3, 5, 7, 9 and 11 were 1680, 1203, 596, 256, 215 and 175 ng/500,000 cells (serum-free) and 21.6, 10.1, 3.6, 2.9, 1.9 and 1.0 ug/500,000 cells (serum-containing), respectively. n = 8.

FIGURE 3  
RESPONSIVENESS TO LH

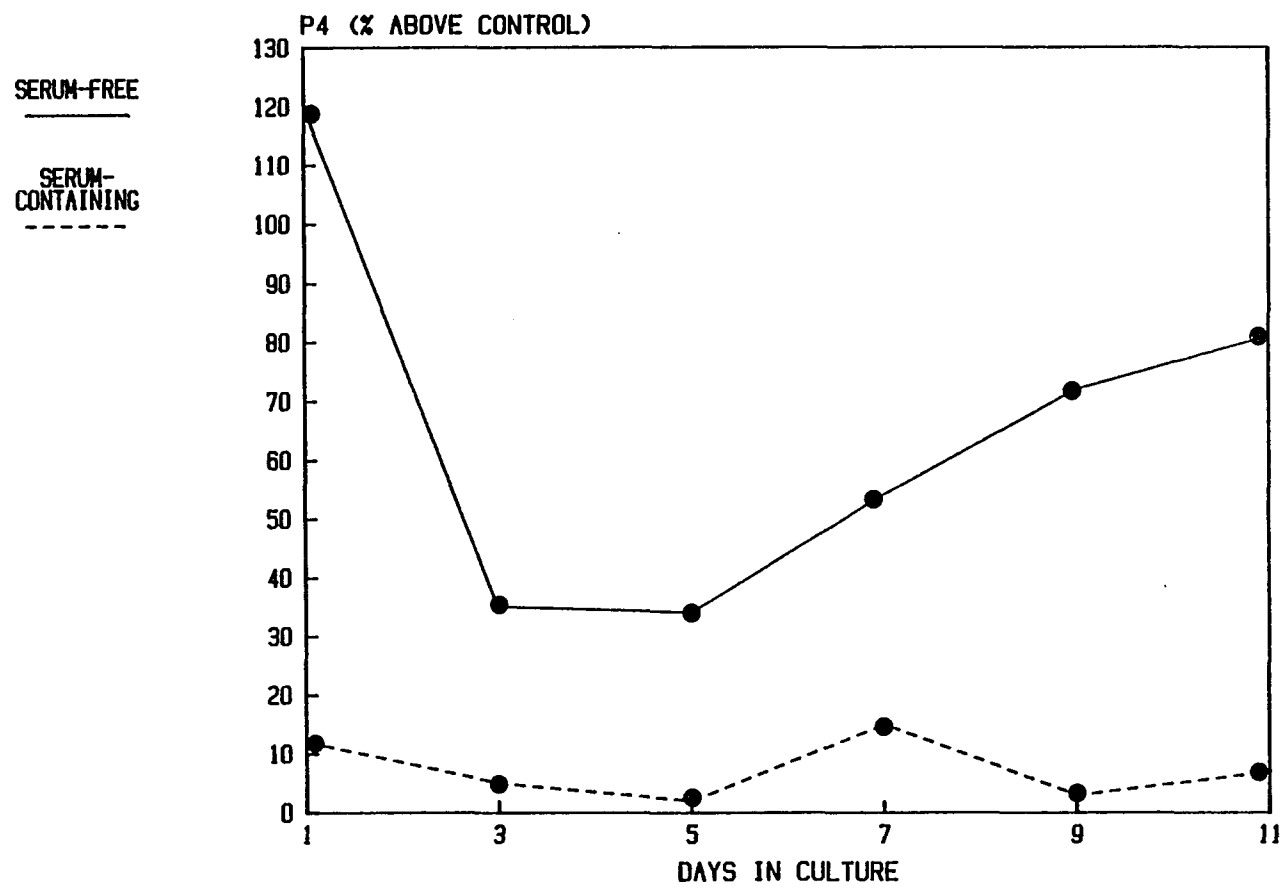


Figure 4. Effect of dbcAMP (1.02 mM) alone and with LH (10 ng/ml) on progesterone production by luteal cells cultured in serum-containing (a) or serum-free (b) medium. Control values for progesterone in Fig. 4a on days 1, 3, 5, 7, 9 and 11 were 4770, 2790, 2065, 1857, 1474 and 1381 ng/500,000 cells, respectively. Comparable values in Fig. 4b were 1560, 1286, 497, 239, 141 and 93 ng/500,000 cells.  $n = 3$ .



FIGURE 4a  
SERUM-CONTAINING MEDIUM

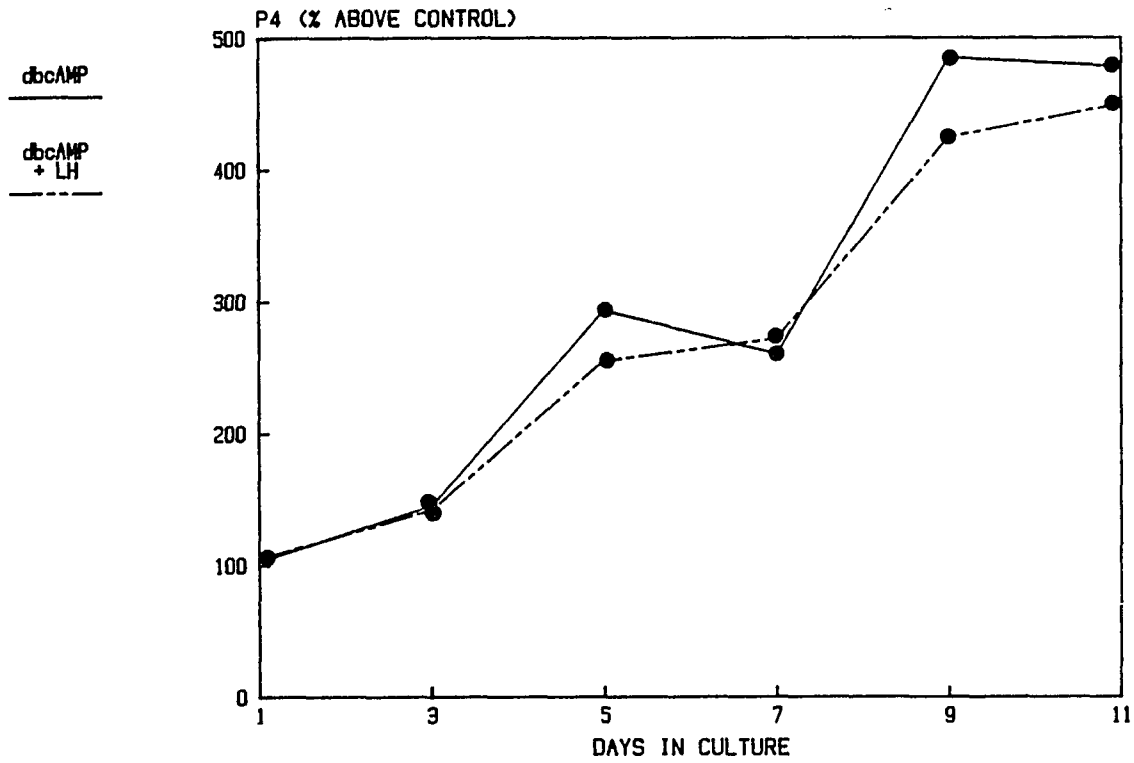


FIGURE 4b  
SERUM-FREE MEDIUM

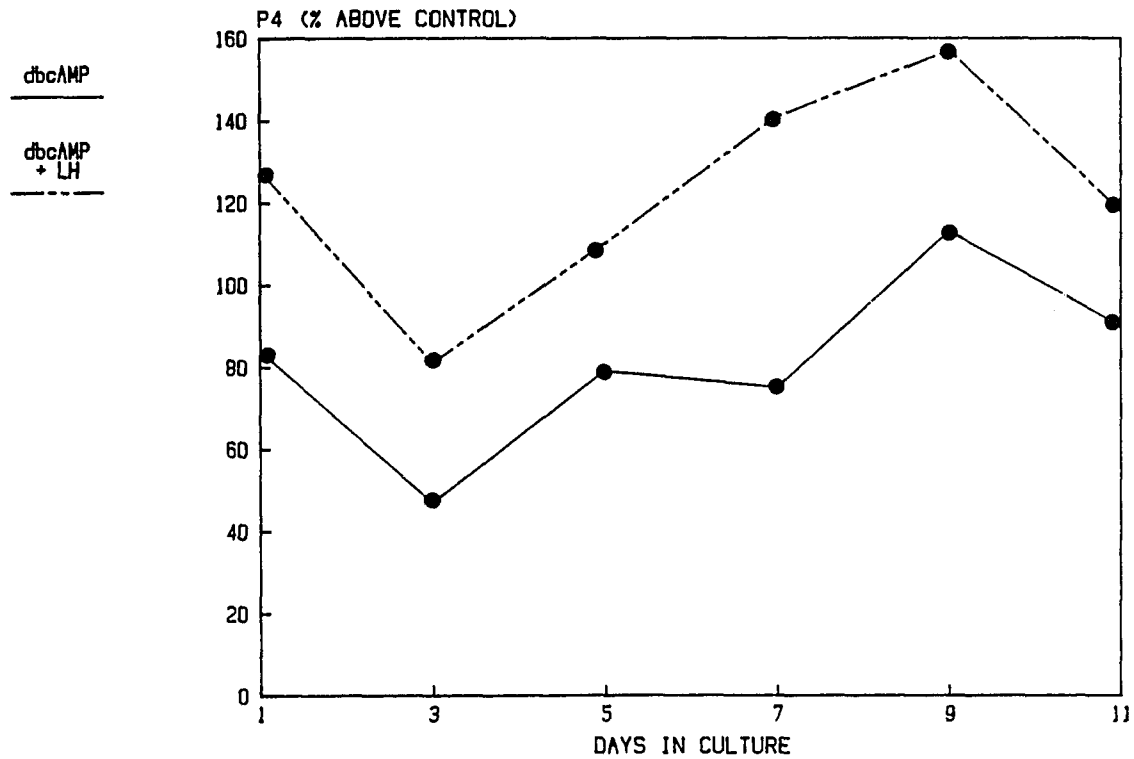


Figure 5. Phase-contrast photomicrograph of bovine luteal cells in serum-containing medium. x195.

Figure 6. Phase-contrast photomicrograph of bovine luteal cells in serum-free medium. x195.

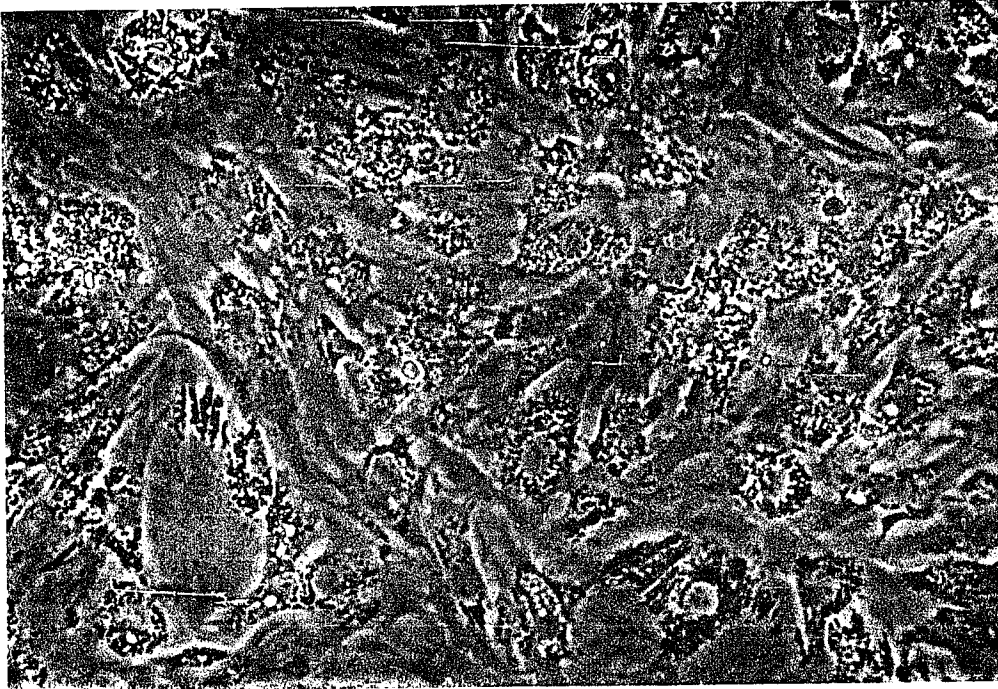


Figure 7. Influence of prolactin (PRL) (10, 50 and 100 ng/ml) on P<sub>4</sub> production alone and with LH (10 ng/ml). Cells were cultured in serum-free medium. Control values for progesterone on days 1, 3, 5, 7, 9 and 11 were 1102, 778, 332, 266, 217 and 151 ng/500,000 cells, respectively. n = 2.

FIGURE 7  
RESPONSE TO PROLACTIN

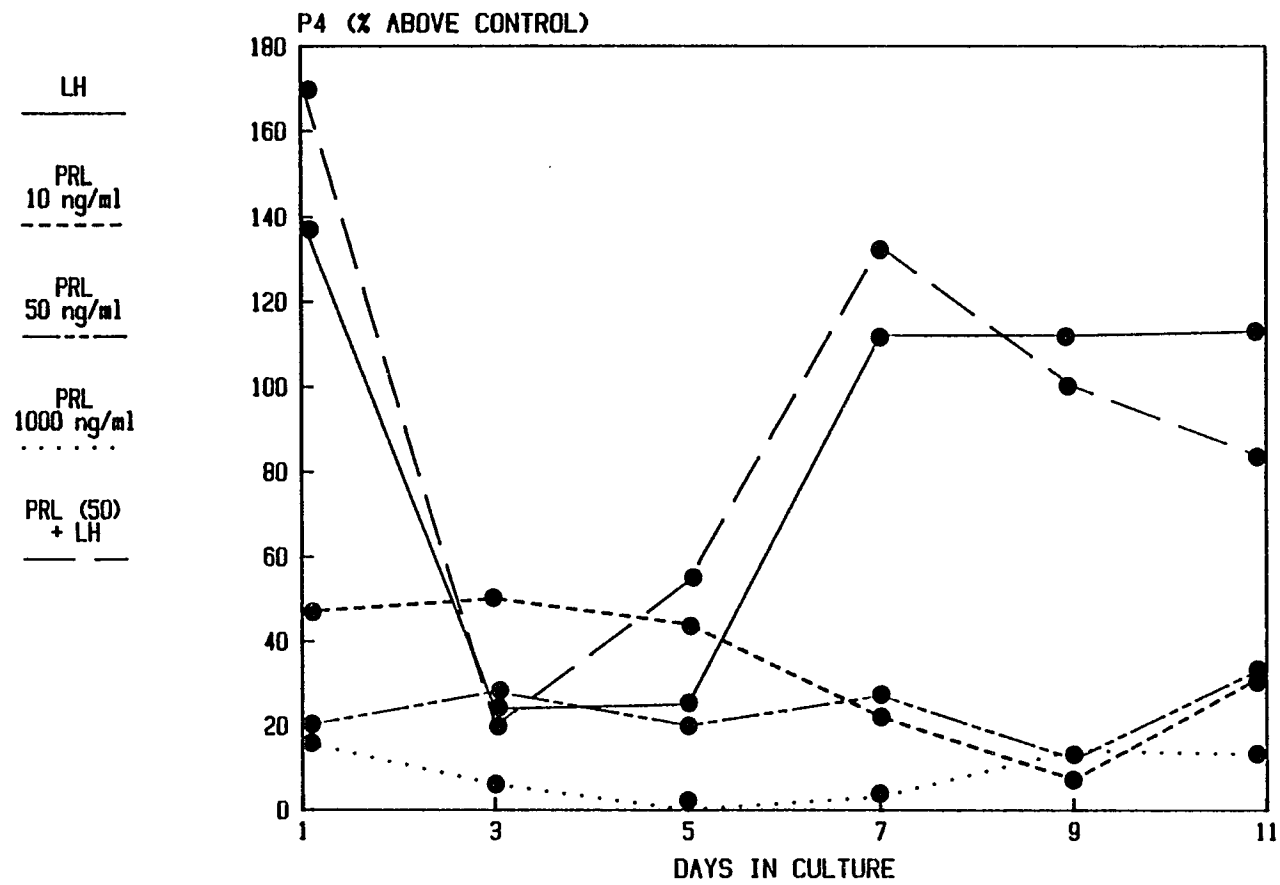


Figure 8. Responsiveness of bovine luteal cells to  $\text{PGE}_1$  during 11 days of culture in serum-free medium. Control values for  $\text{P}_4$  on days 1, 3, 5, 7, 9 and 11 were 2012, 1326, 865, 401 and 310 ng/500,000 cells, respectively.  $n = 5$ .

FIGURE 8  
RESPONSE TO PGE-1

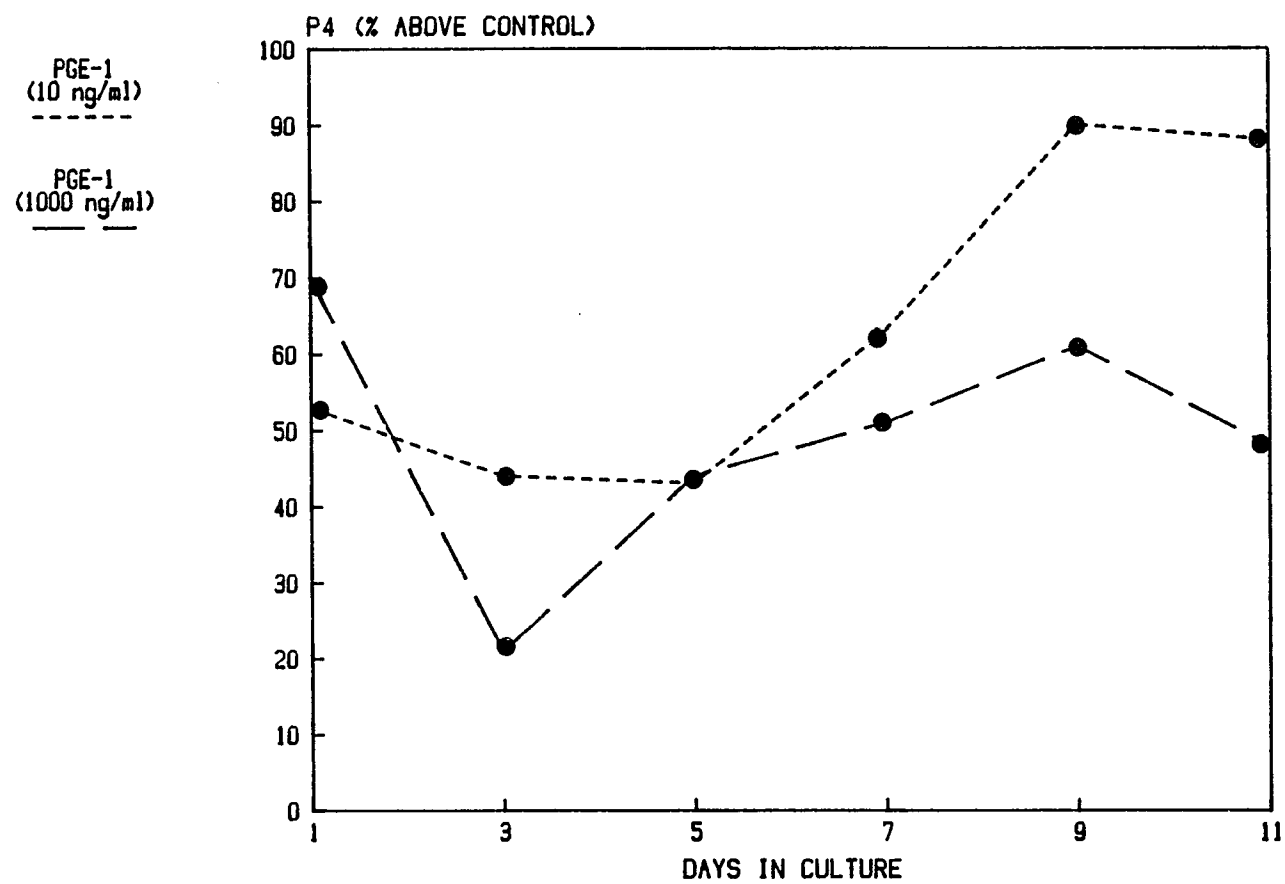


Figure 9. Effect of cell density on the ability of luteal cells to respond to LH.  $n = 2$ .



FIGURE 9  
CELL DENSITY

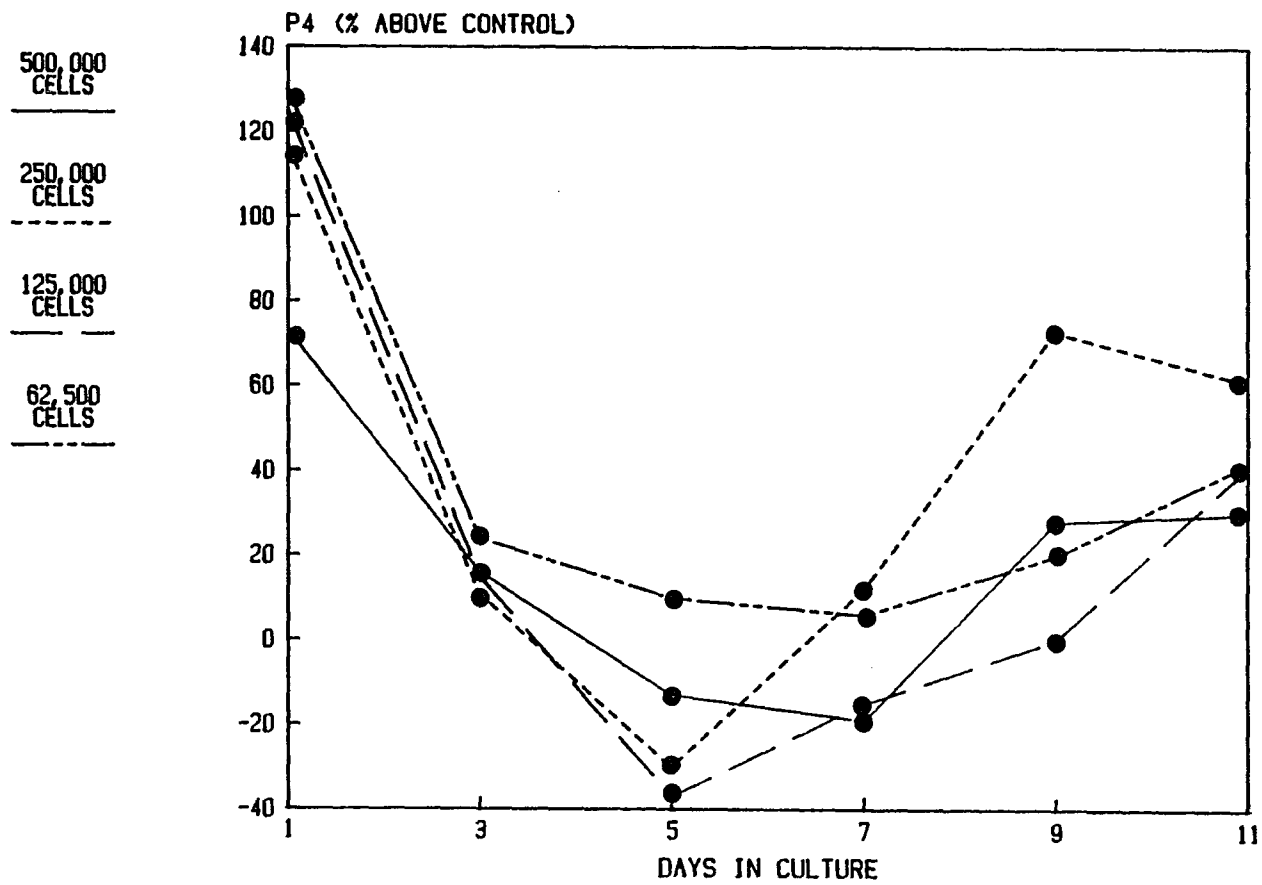


Figure 10. Effect of LDL and HDL on luteal progesterone production. Cells were cultured in serum-free medium either with or without the addition of LDL or HDL (50 ug cholesterol/ml) as described in detail in the text (results). Mean  $\pm$  SEM, n = 7 for days 5-9, n = 2 for day 1 and 3. Values different from control are indicated: \*P<0.05, \*\*P<0.01, \*\*\*P<0.001.

FIGURE 10

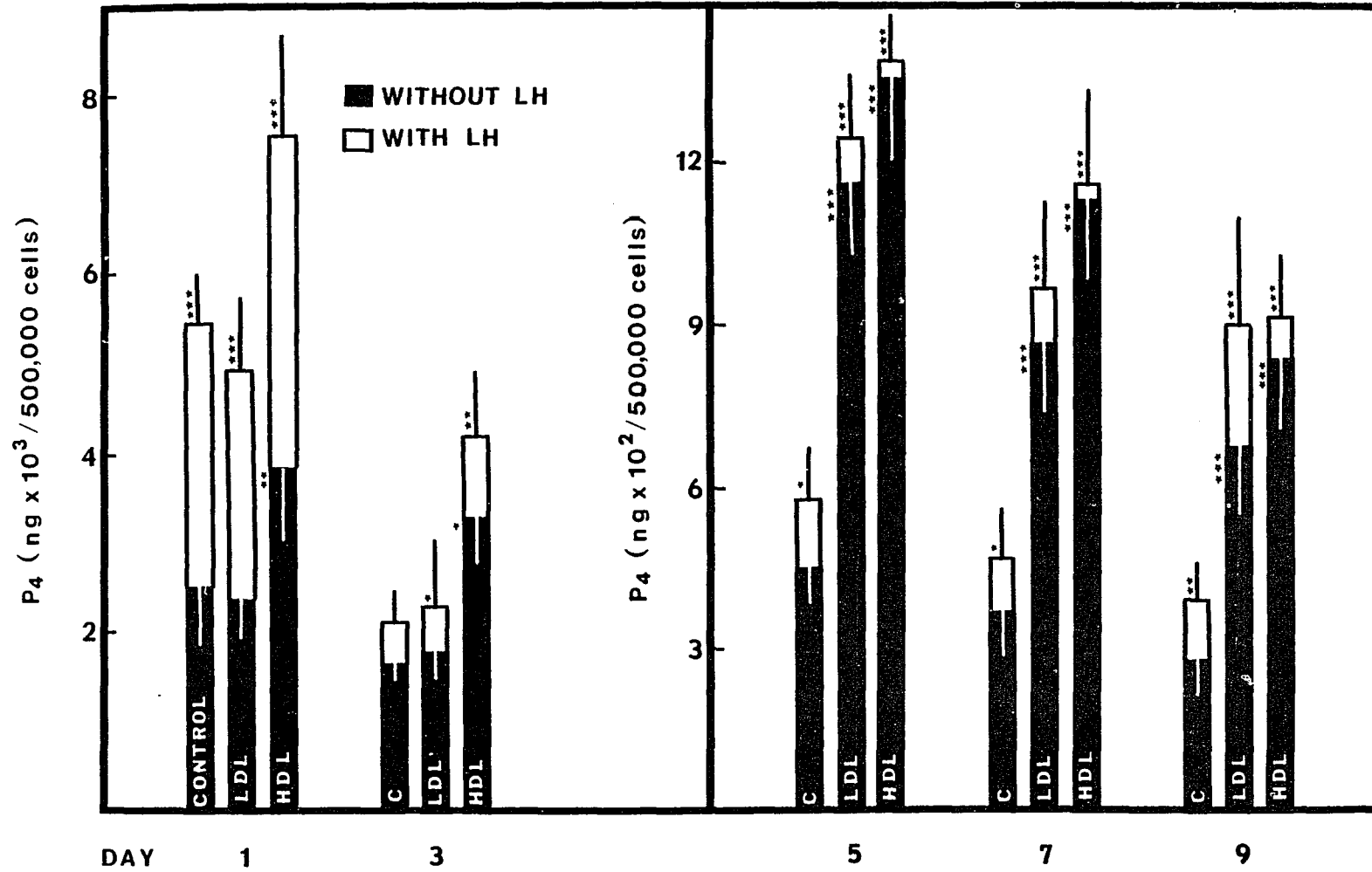


Figure 11.  $^{14}\text{C}$ -acetate incorporation into cholesterol on day 2 of culture - effect of LDL and HDL (50 ug cholesterol/ml). Culture conditions and experimental procedures were as described in materials and methods. Mean  $\pm$  SD, n = 3.

FIGURE 11  
LIPOPROTEINS & STEROL SYNTHESIS

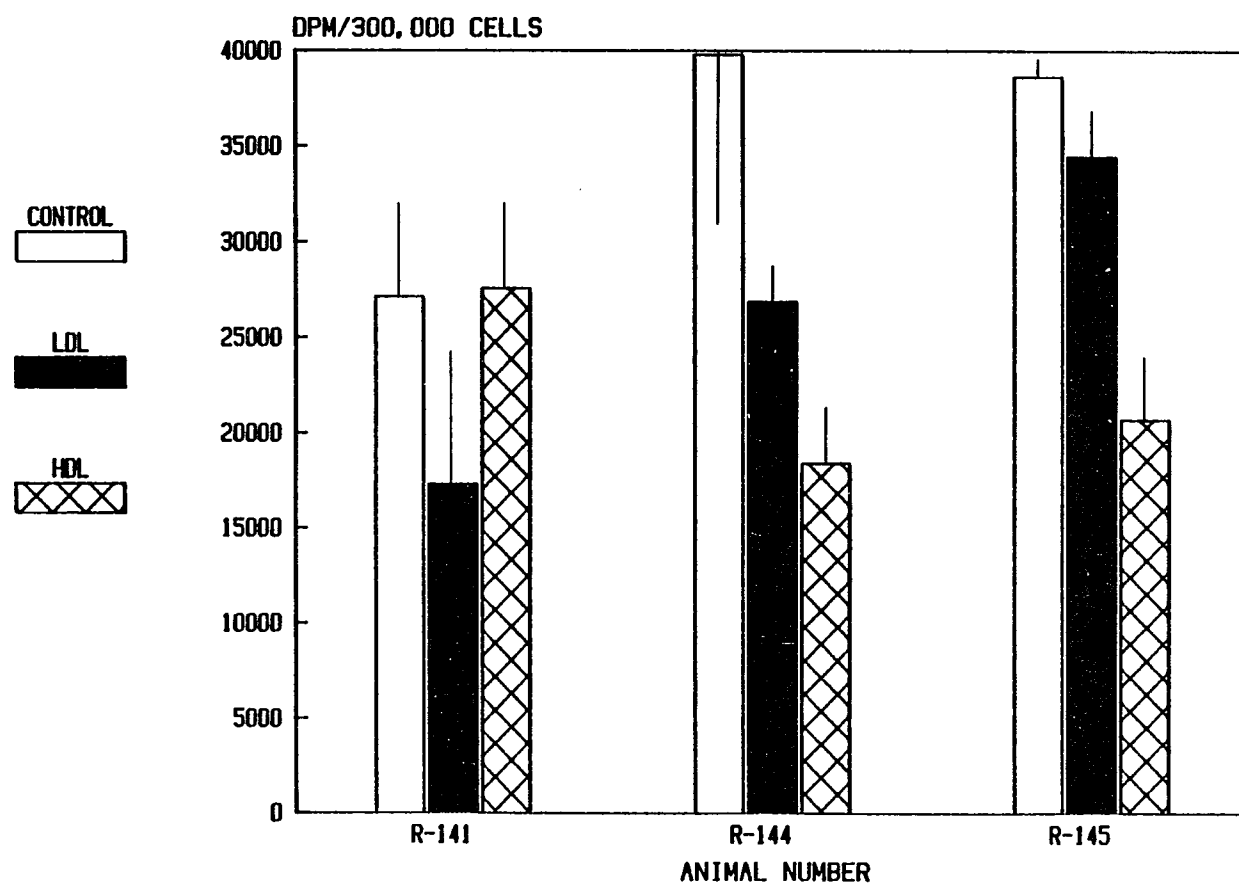


Figure 12.  $^{14}\text{C}$ -acetate incorporation into cholesterol on day 6 of culture - effects of LDL and HDL (50 ug cholesterol/ml). Mean  $\pm$  SD, n = 3.

FIGURE 12  
LIPOPROTEINS & STEROL SYNTHESIS

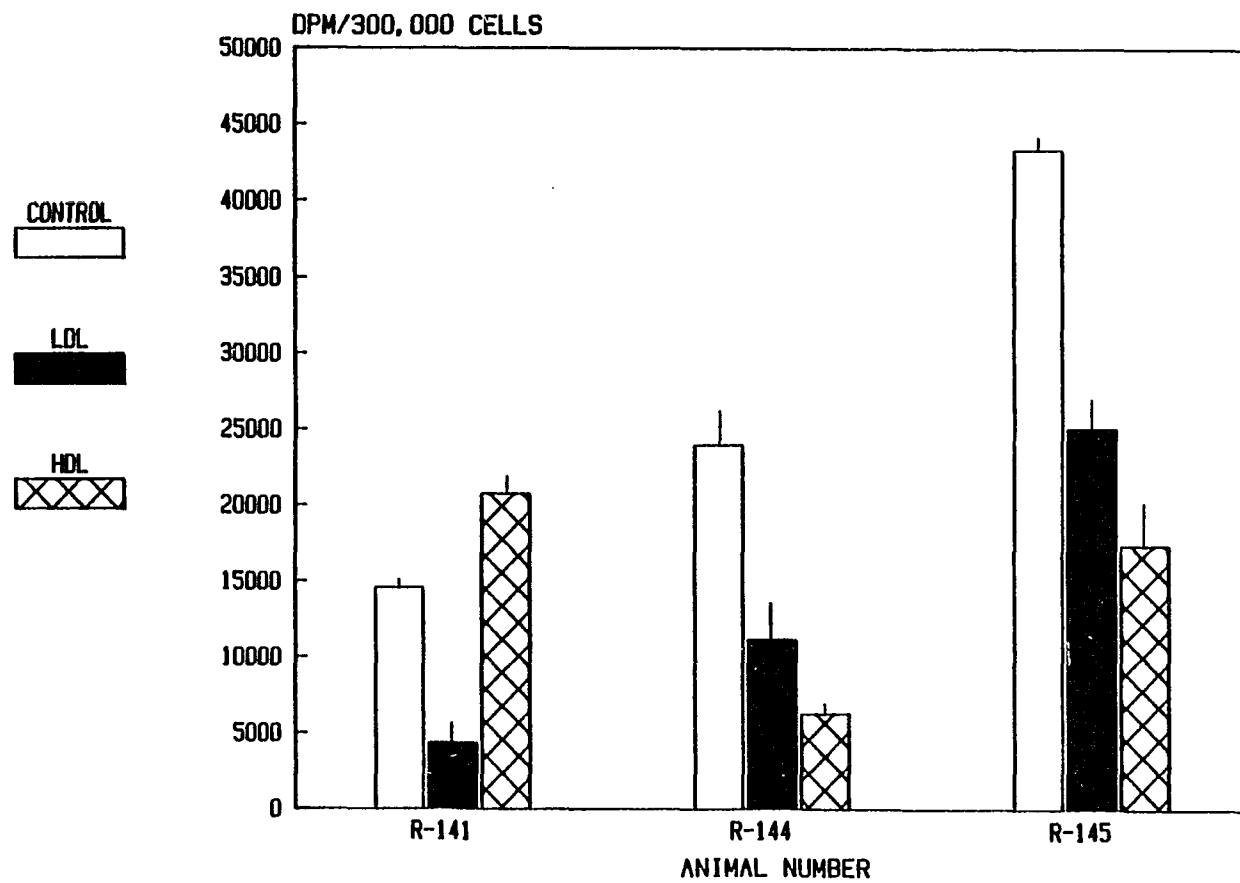


Figure 13.  $^{14}\text{C}$ -acetate incorporation into cholesterol on day 11 of culture - effects of LDL and HDL (50 ug cholesterol/ml). Mean  $\pm$  SD, n = 3.



FIGURE 13  
LIPOPROTEINS & STEROL SYNTHESIS

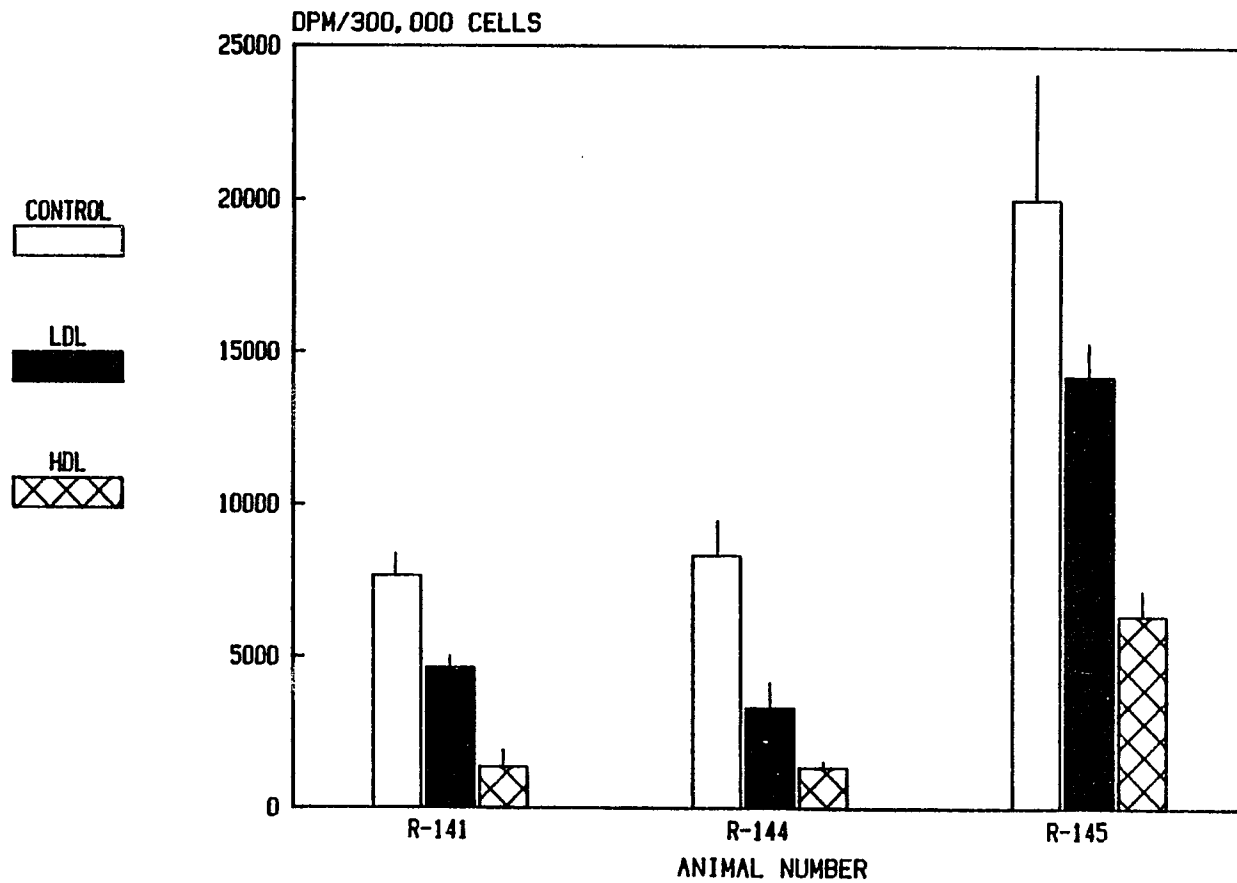


Figure 14. Inhibition of  $P_4$  synthesis from acetate in the presence of LDL and HDL (50 ug cholesterol/ml). Mean  $\pm$  SEM, n = 3.

FIGURE 14  
SYNTHESIS OF P4 FROM ACETATE

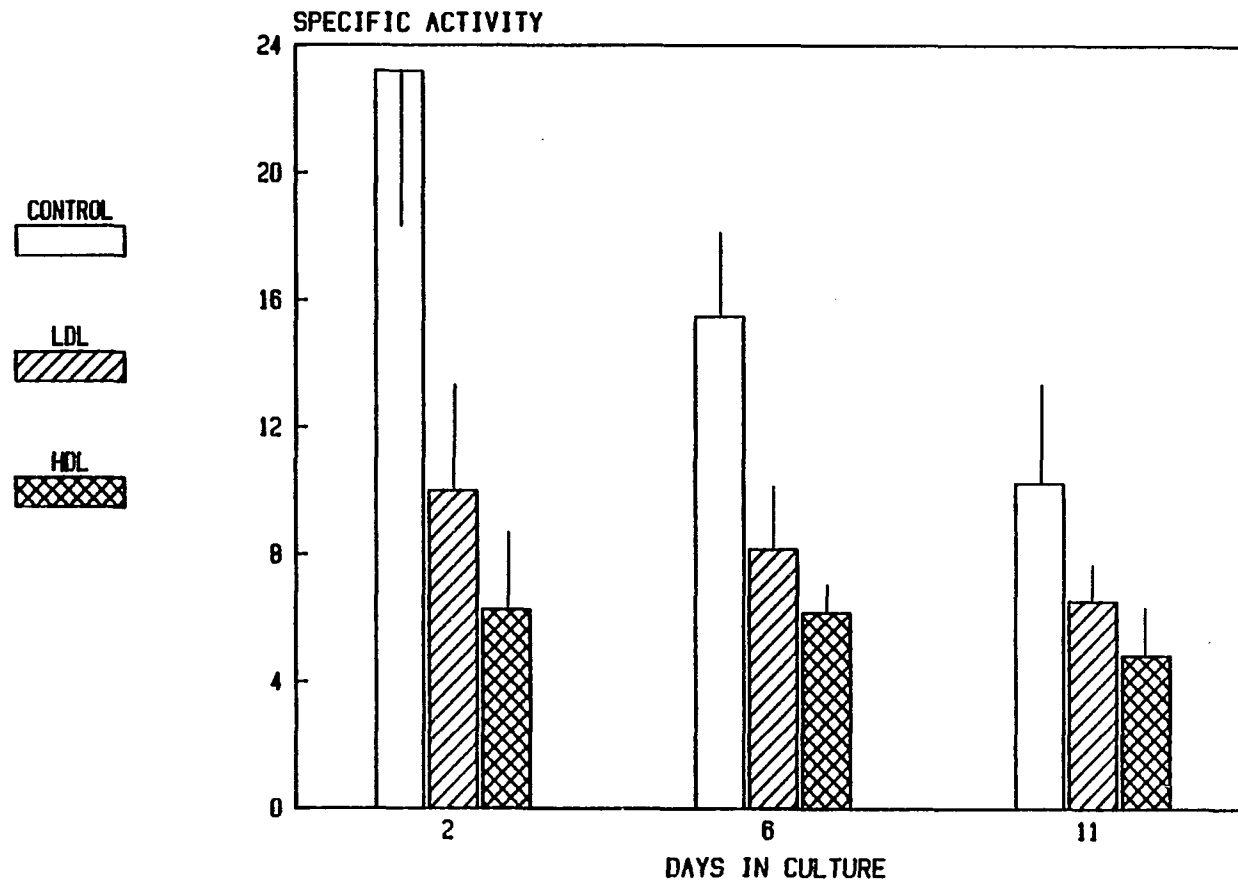


Figure 15. Effect of LH (10 ng/ml) and LH + LDL or HDL (50 ug cholesterol/ml) on synthesis of  $P_4$  from acetate. Mean  $\pm$  SEM, n = 3.

FIGURE 15  
SYNTHESIS OF P4 FROM ACETATE

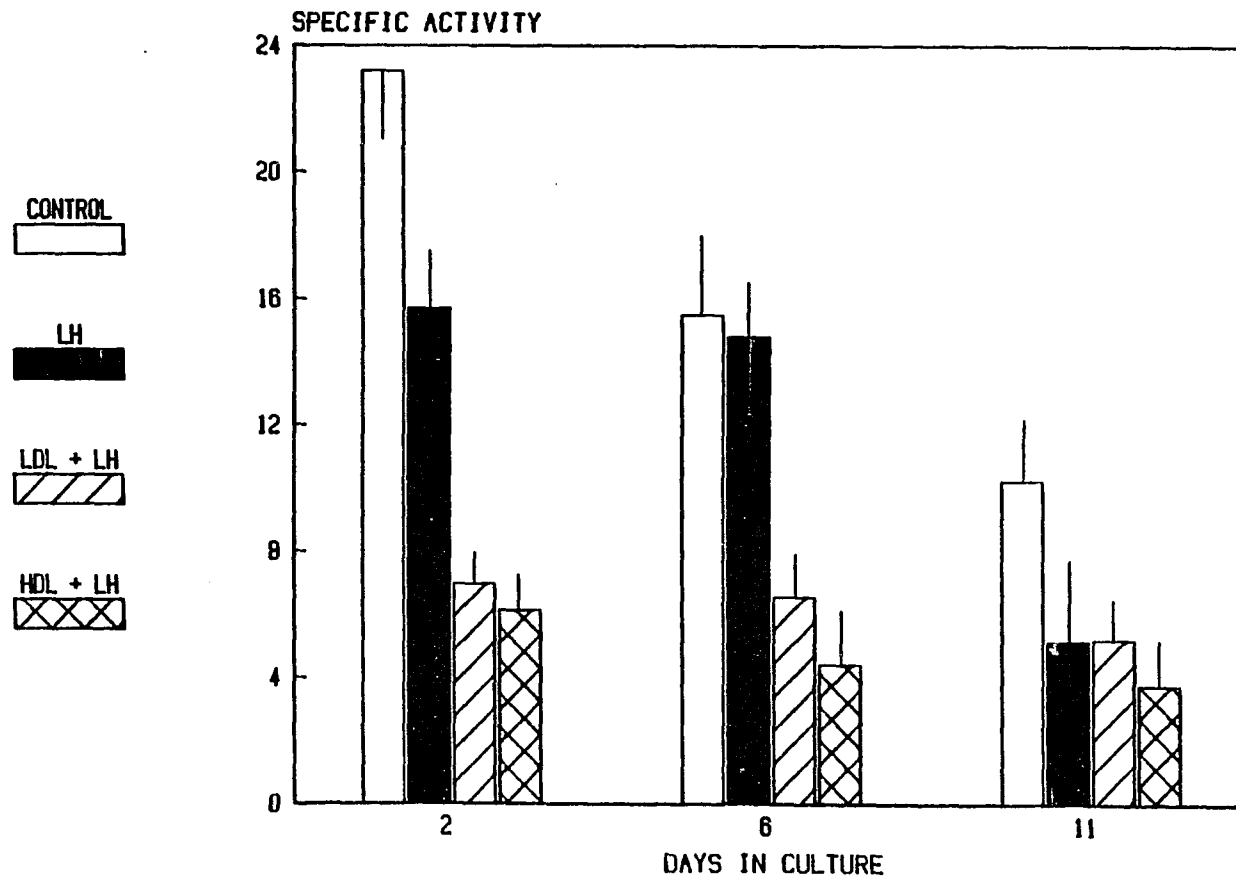


Figure 16. Synthesis of 6-keto-PGF<sub>1 $\alpha$</sub>  by cultured bovine luteal cells. Cells were cultured for 1, 5, or 11 days either alone, in the presence of LH (10 ng/ml), indomethacin (INDO, 10 ug/ml) or LH + INDO. Bars within a group with different superscripts are statistically different ( $P < 0.01$ ). Mean  $\pm$  SEM,  $n = 3$ .

FIGURE 16  
6-KETO-PGF-1-alpha

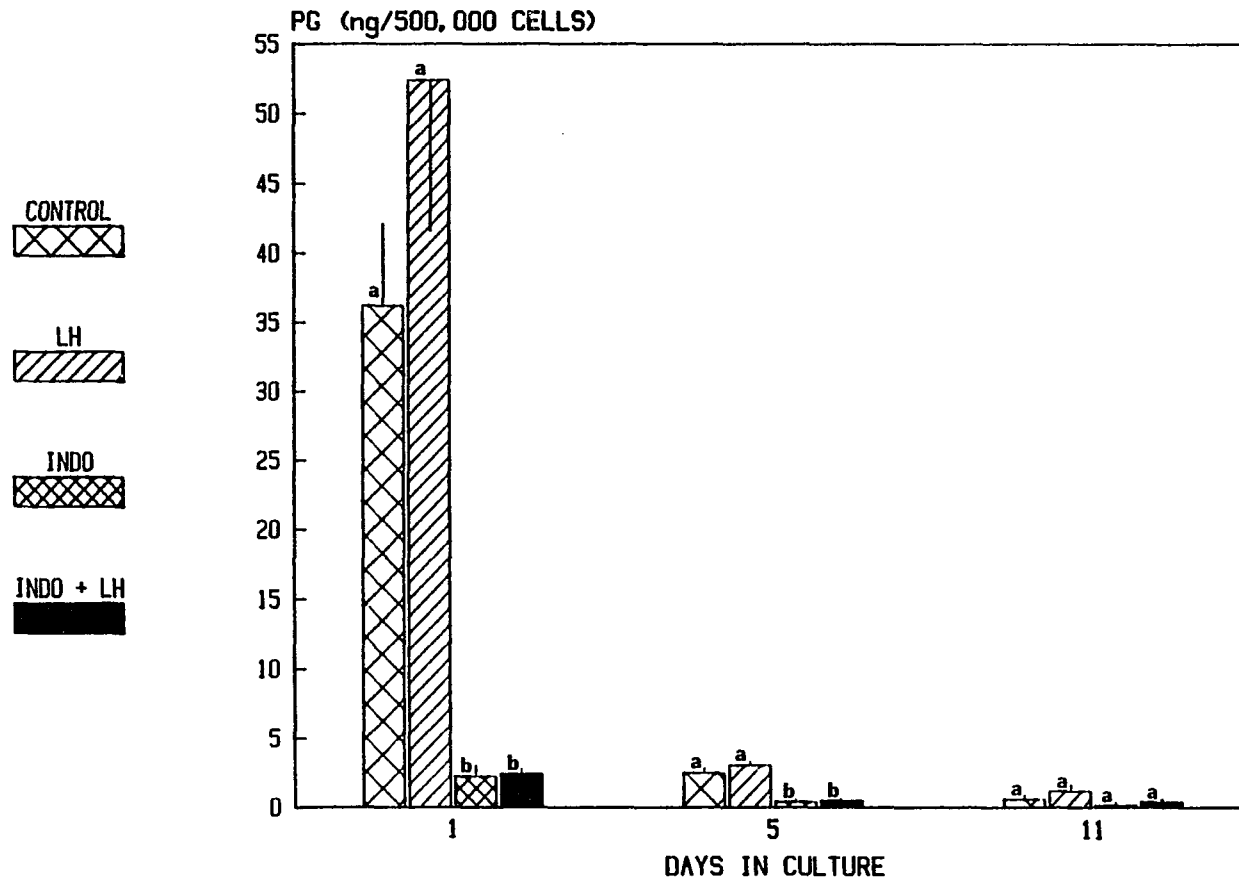


Figure 17. Synthesis of  $P_4$  in response to LH (10 ng/ml),  $PGF_{2\alpha}$  (PGF, 10 and 100 ng/ml) and LH +  $PGF_{2\alpha}$  on days 1, 3 and 5 of culture. Bars within a group with different superscripts are statistically different ( $P < 0.05$ , day 1;  $P < 0.01$ , day 5). Mean  $\pm$  SEM,  $n = 5$ .



FIGURE 17

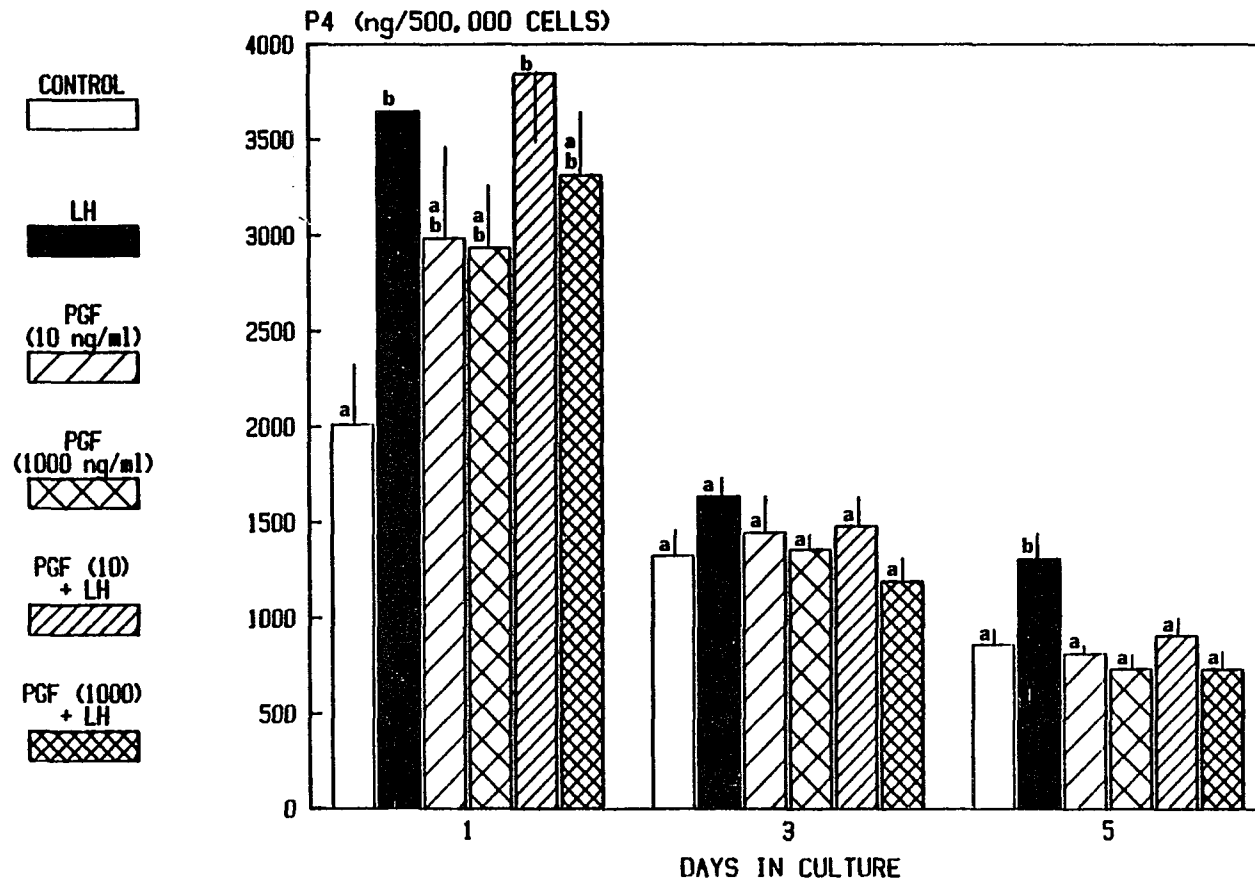


Figure 18. Synthesis of  $P_4$  in response to LH (10 ng/ml),  $PGF_{2\alpha}$  (PGF, 10 and 100 ng/ml) and LH +  $PGF_{2\alpha}$  on days 7, 9 and 11 of culture. Bars within a group with different superscripts are statistically different ( $P < 0.001$ , day 7;  $P < 0.05$ , day 9). Mean  $\pm$  SEM,  $n = 5$ .

FIGURE 18

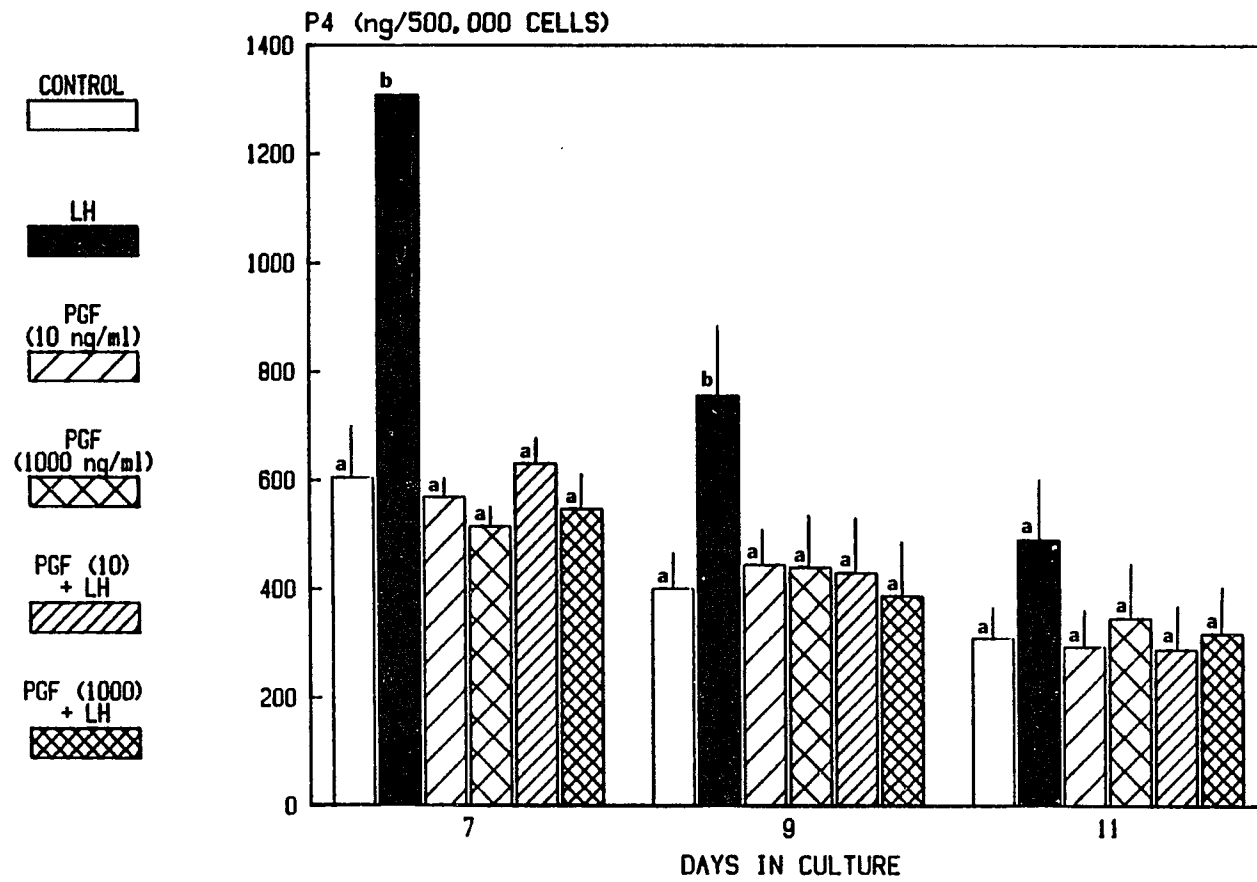


Figure 19. Mechanism of action of  $\text{PGF}_{2\alpha}$  - day 1 of culture. Cells were treated with either LH (10 ng/ml), forskolin (FKN, 10 mM) or indomethacin (INDO, 10 ug/ml), alone and in combination with  $\text{PGF}_{2\alpha}$  (PGF, 10 ng/ml). C = control (solid line). Bars within a group with different superscripts are statistically different ( $P < 0.05$ , LH and CT;  $P < 0.01$ , FKN). Mean  $\pm$  SEM,  $n = 5$ .

FIGURE 19  
DAY 1 OF CULTURE

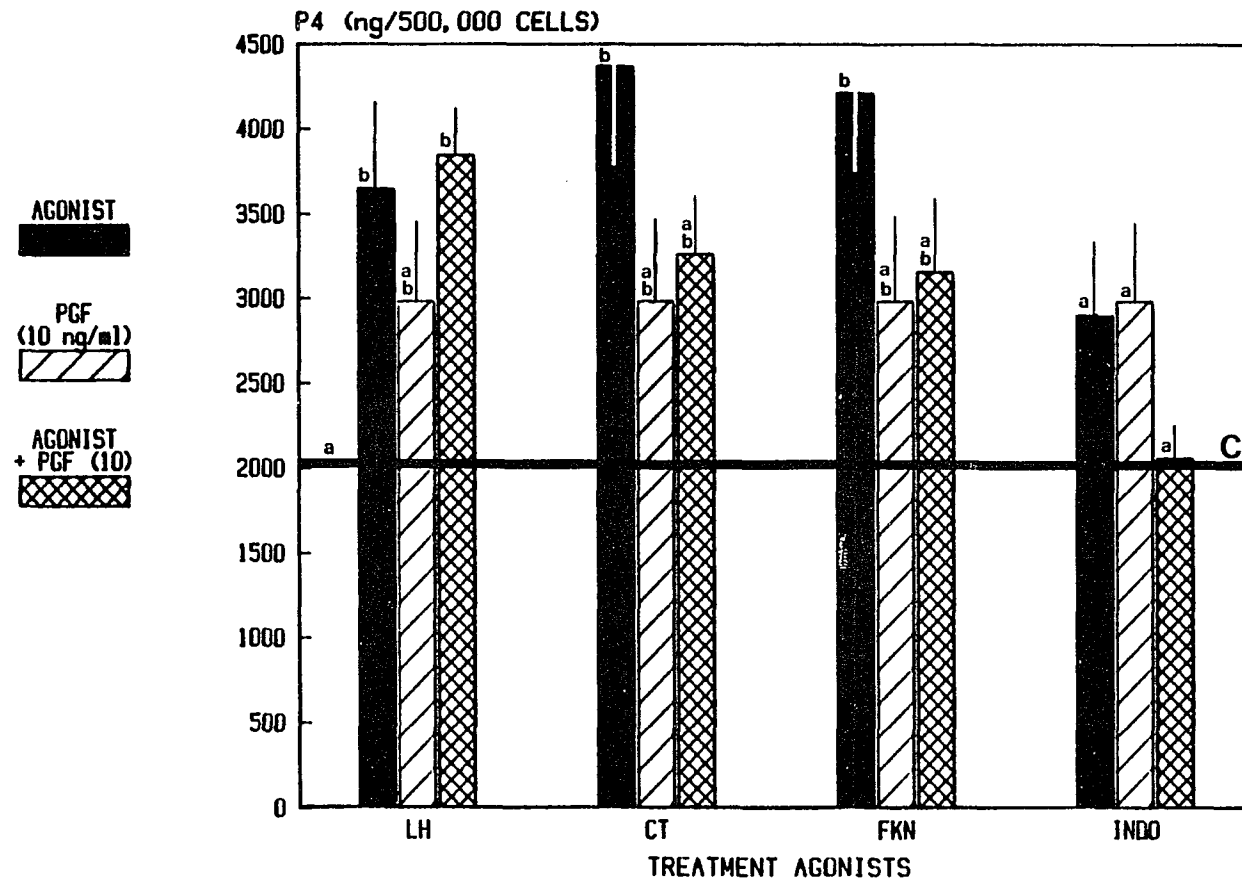


Figure 20. Mechanism of action of  $\text{PGF}_{2\alpha}$  - day 3 of culture. Treatments were as described in Fig. 19. Bars within a group with different superscripts are statistically different ( $P < 0.01$ , FKN and INDO;  $P < 0.001$ , CT). Mean  $\pm$  SEM,  $n = 5$ .

FIGURE 20  
DAY 3 OF CULTURE

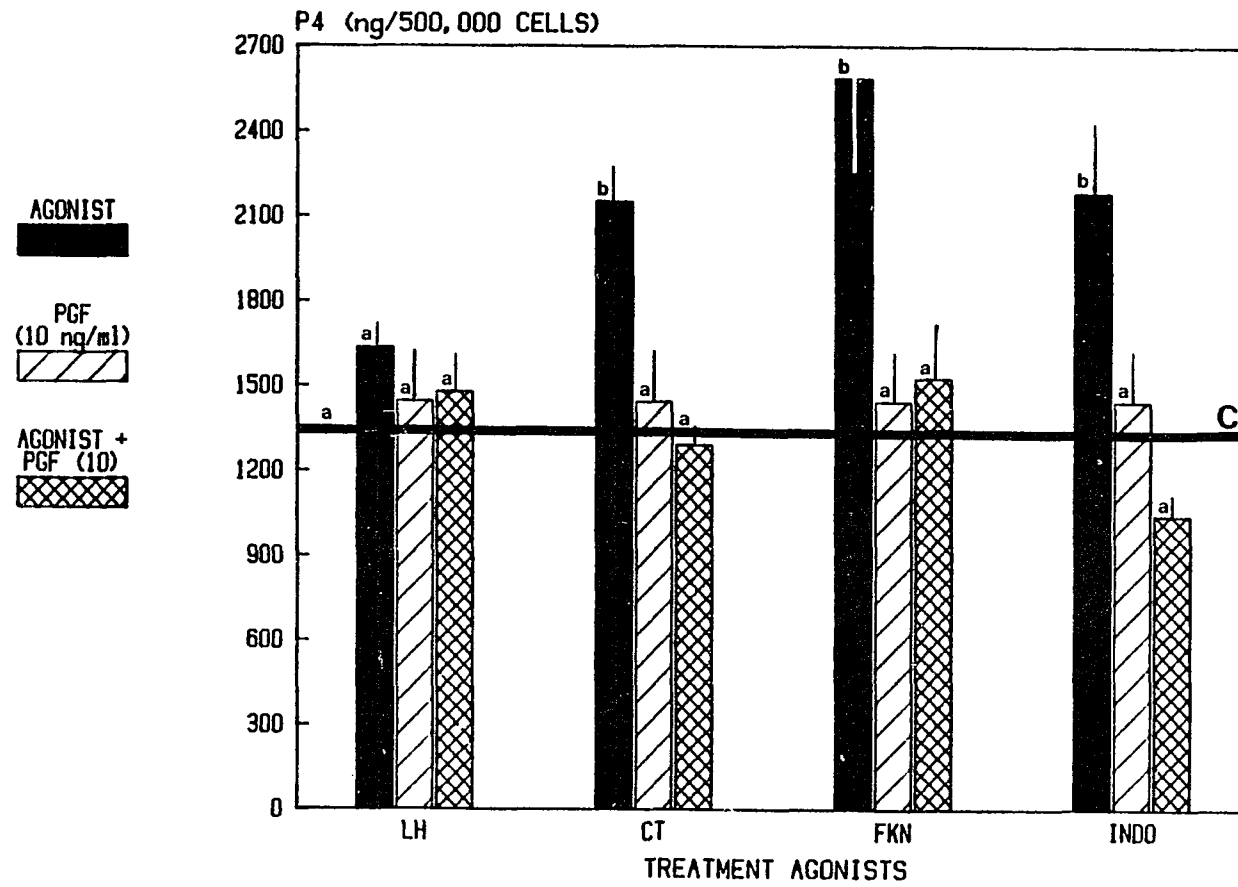


Figure 21. Mechanism of action of  $\text{PGF}_{2\alpha}$  - day 5 of culture. Treatments were as described in Fig. 19. Bars within a group with different superscripts are statistically different ( $P < 0.01$ , LH;  $P < 0.001$ , CT, FKN, IND0). Mean  $\pm$  SEM,  $n = 5$ .



FIGURE 21  
DAY 5 OF CULTURE

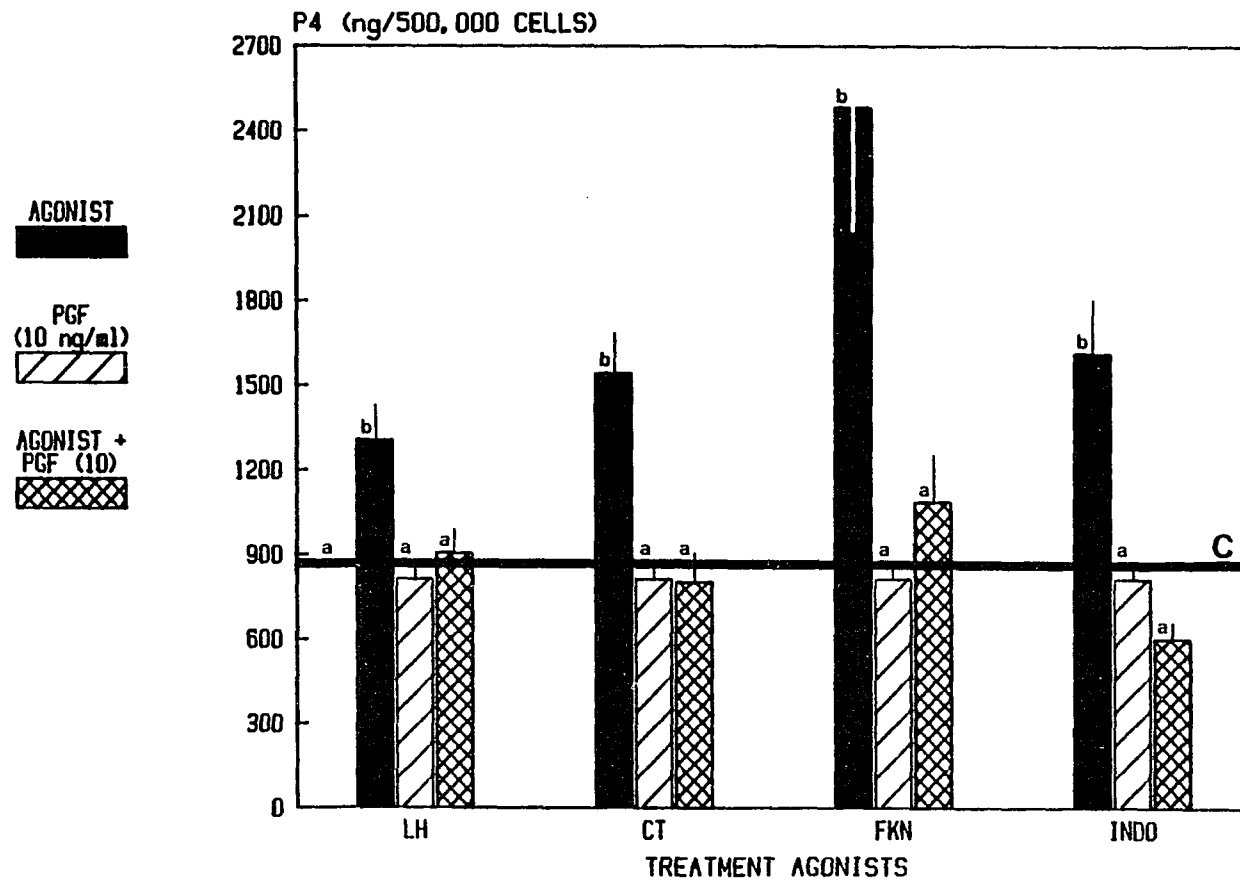


Figure 22. Mechanism of action of  $\text{PGF}_{2\alpha}$  - day 7 of culture. Treatments were as described in Fig. 19. Bars within a group with different superscripts are statistically different ( $P < 0.001$ ). Mean  $\pm$  SEM,  $n = 5$ .

FIGURE 22  
DAY 7 OF CULTURE

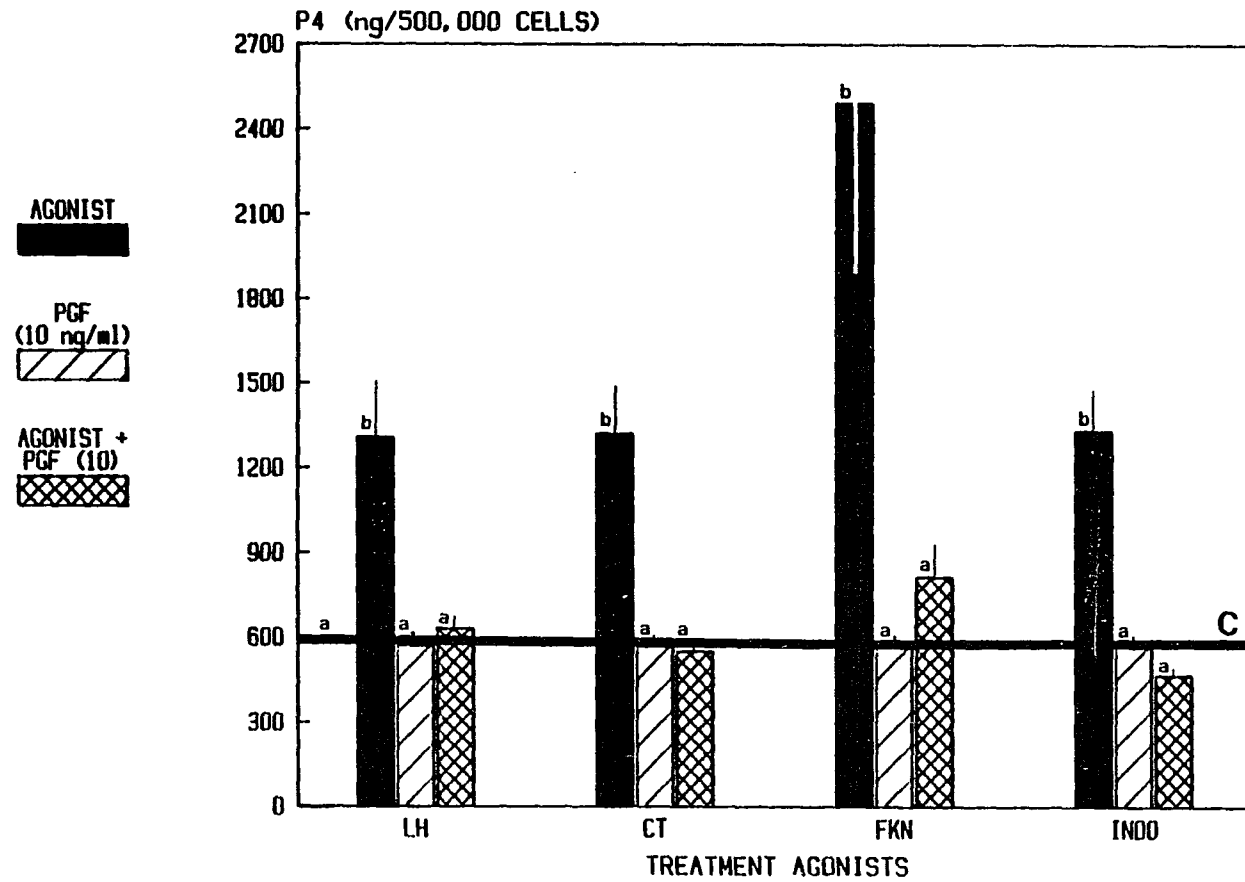


Figure 23. Mechanism of action of  $\text{PGF}_{2\alpha}$  - day 9 of culture. Treatments were as described in Fig. 19. Bars within a group with different superscripts are statistically different ( $P < 0.05$ , LH, CT;  $P < 0.01$ , FKN, INDO). Mean  $\pm$  SEM,  $n = 5$ .

FIGURE 23  
DAY 9 OF CULTURE

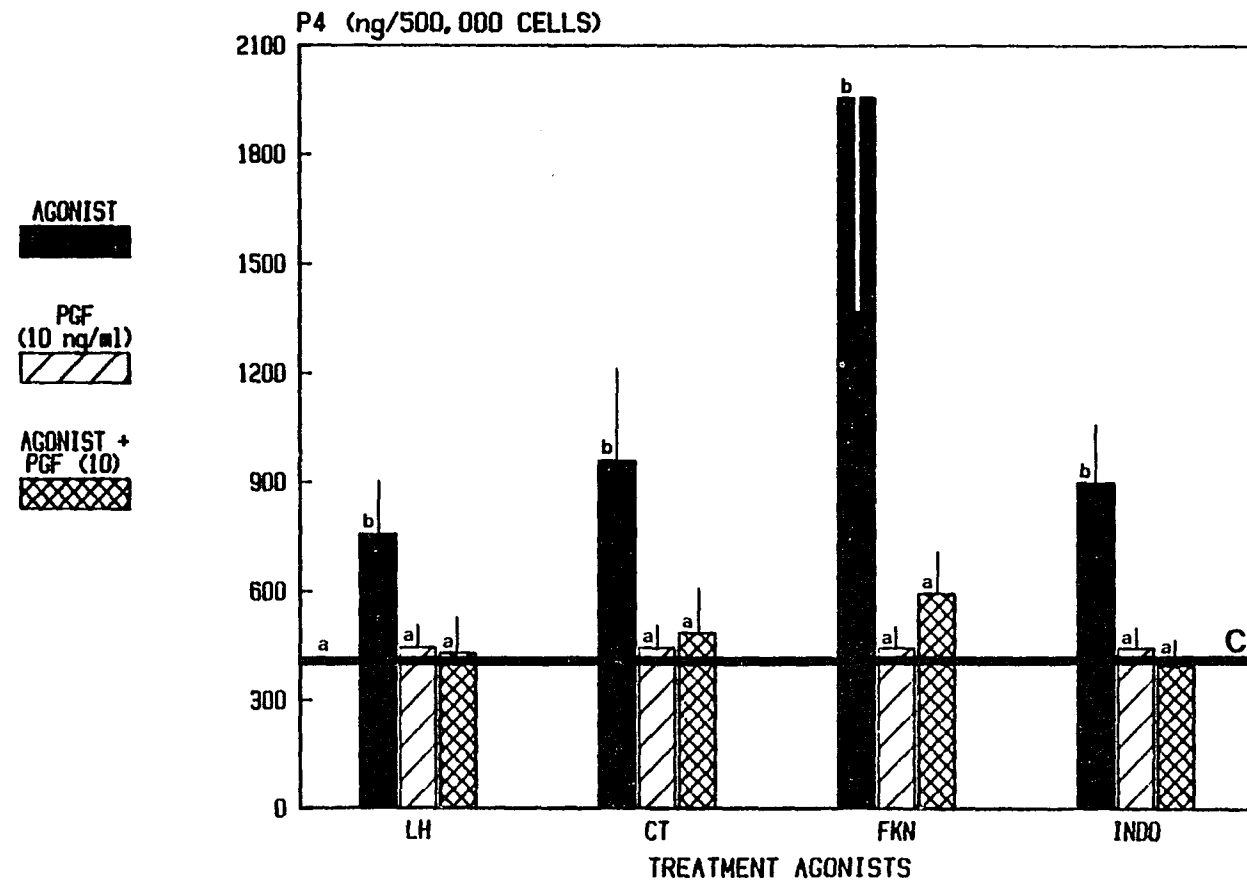


Figure 24. Mechanism of action of  $\text{PGF}_{2\alpha}$  - day 11 of culture. Treatments were as described in Fig. 19. Bars within a group with different superscripts are statistically different ( $P < 0.05$ ). Mean  $\pm$  SEM,  $n = 5$ .

FIGURE 24  
DAY 11 OF CULTURE

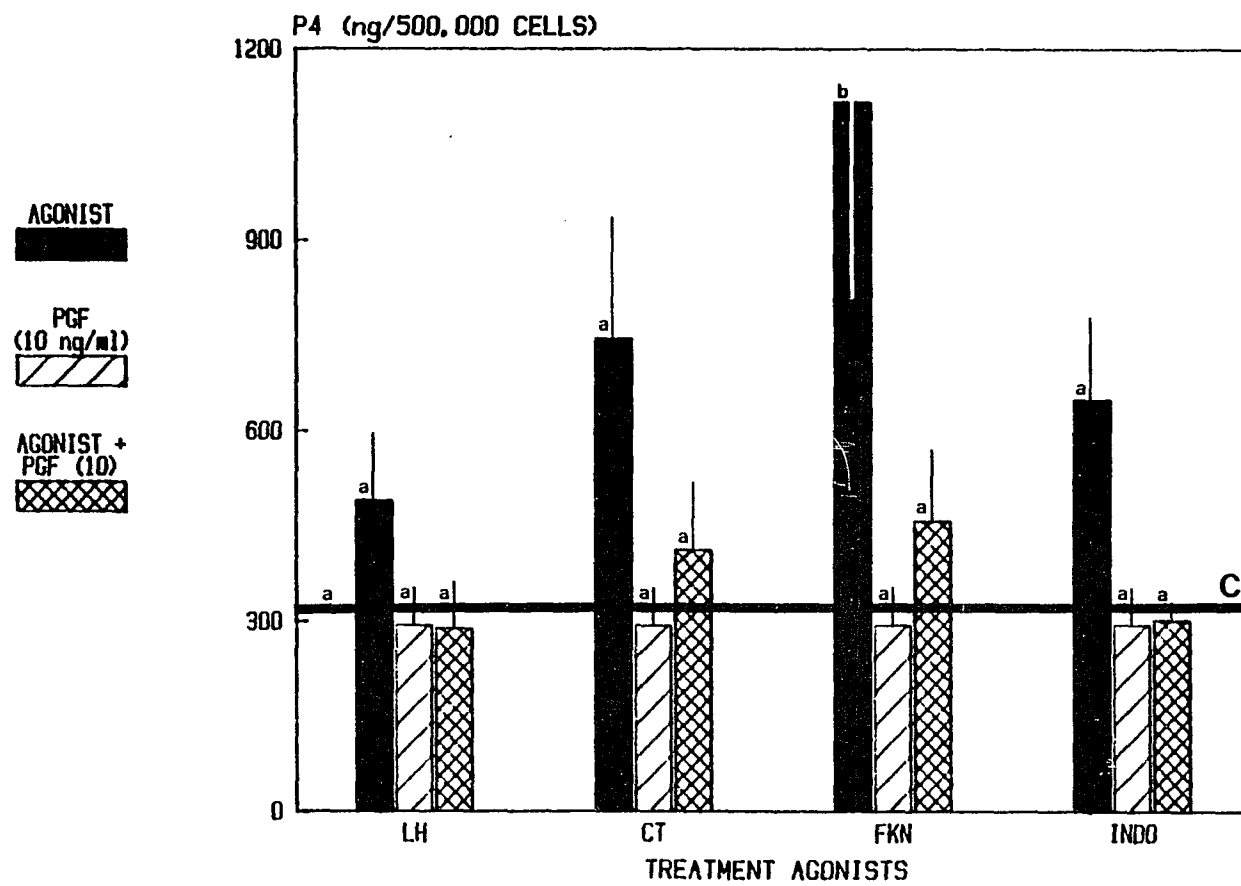


Figure 25. Interaction of hCG and dbcAMP with  $\text{PGF}_{2\alpha}$  - day 1 of culture. Cells were treated with either hCG (10 ng/ml) or dbcAMP (1mM), alone and in combination with  $\text{PGF}_{2\alpha}$  (PGF, 10 ng/ml). Bars within a group with different superscripts are statistically different ( $P < 0.05$ ). Mean  $\pm$  SEM, dbcAMP, n = 3; hCG, n = 2.



FIGURE 25  
DAY 1 OF CULTURE

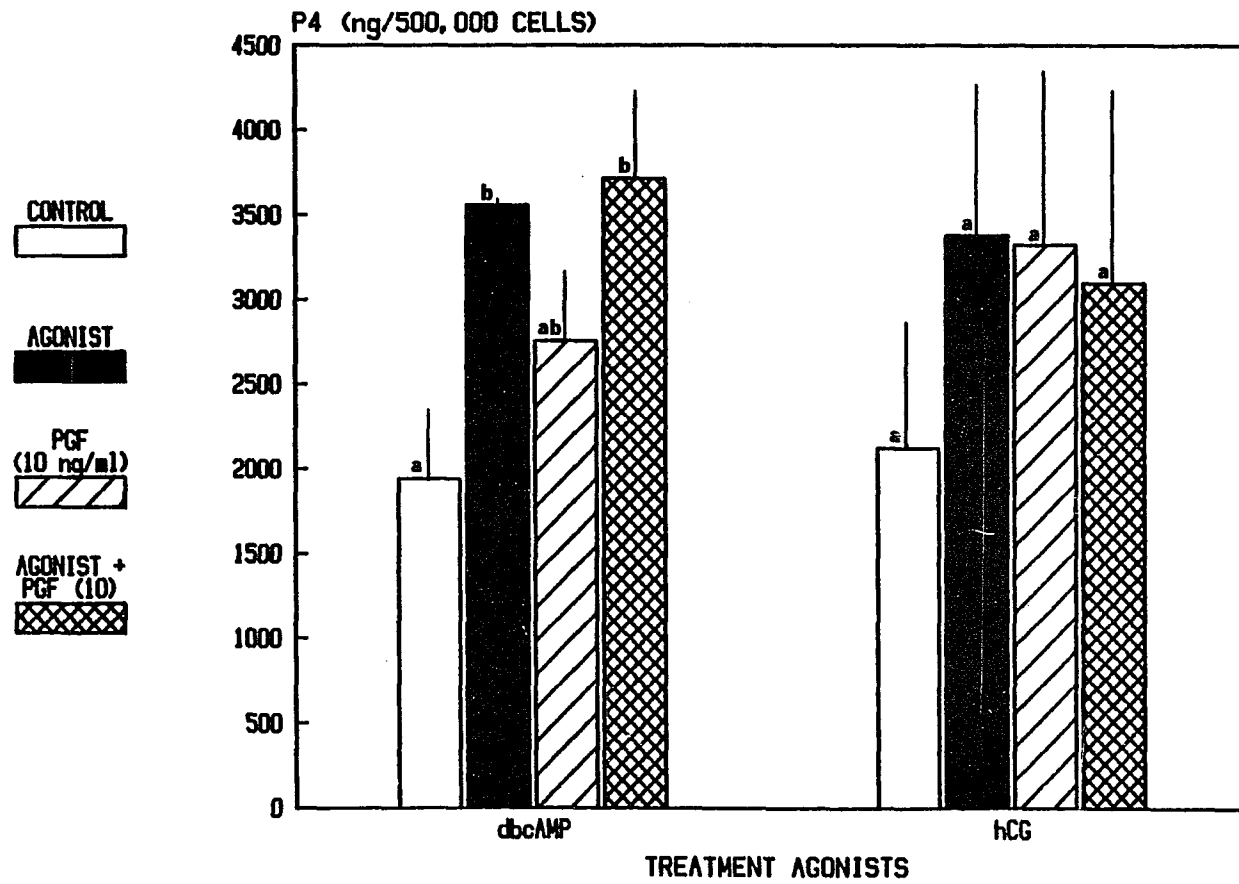


Figure 26. Interaction of hCG and dbcAMP with  $\text{PGF}_{2\alpha}$  - day 5 of culture. Treatments were as described in Fig. 25. Bars within a group with different superscripts are statistically different ( $P < 0.01$ ). Mean  $\pm$  SEM, dbcAMP,  $n = 3$ ; hCG,  $n = 2$ .

FIGURE 26  
DAY 5 OF CULTURE

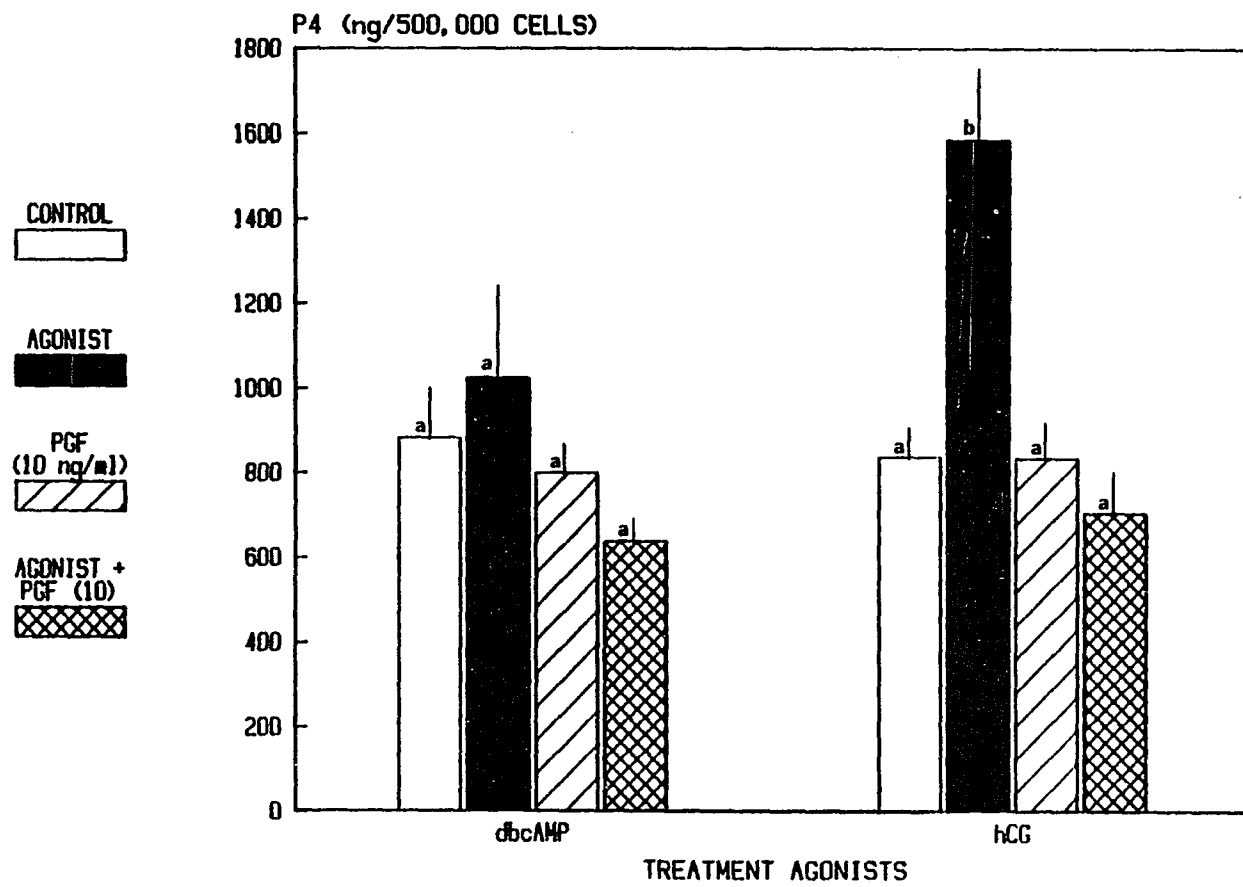


Figure 27. Interaction of hCG and dbcAMP with  $\text{PGF}_{2\alpha}$  - day 9 of culture. Treatments were as described in Fig. 25. Bars within a group with different superscripts are statistically different ( $P < 0.01$ ). Mean  $\pm$  SEM, dbcAMP,  $n = 3$ ; hCG,  $n = 2$ .

FIGURE 27  
DAY 9 OF CULTURE

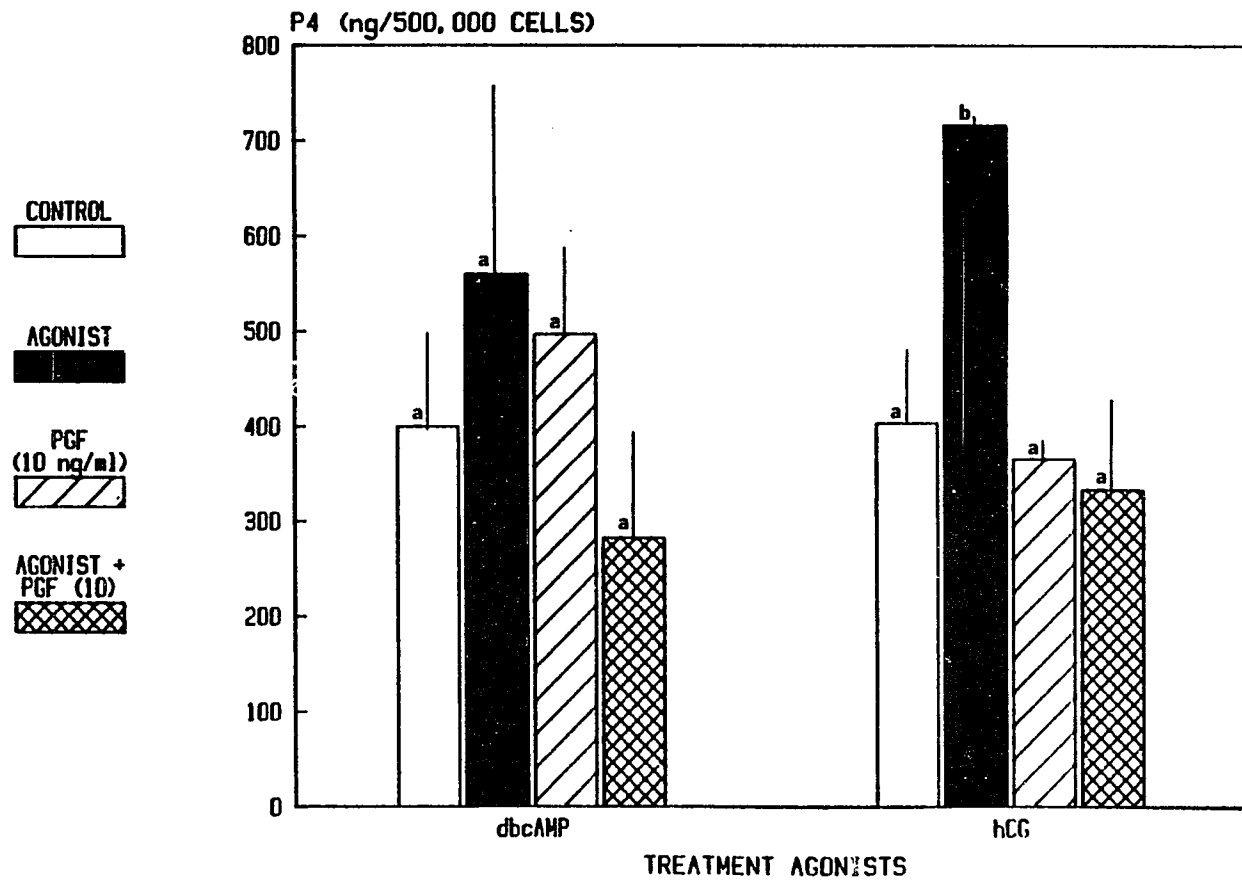


Figure 28. Phase-contrast photomicrograph of bovine luteal cells cultured for 11 days in serum-free medium, with no hormone additions. x195.

Figure 29. Phase-contrast photomicrograph of bovine luteal cells cultured for 11 days in serum-free medium with the addition of LH. (10 ng/ml). x195.

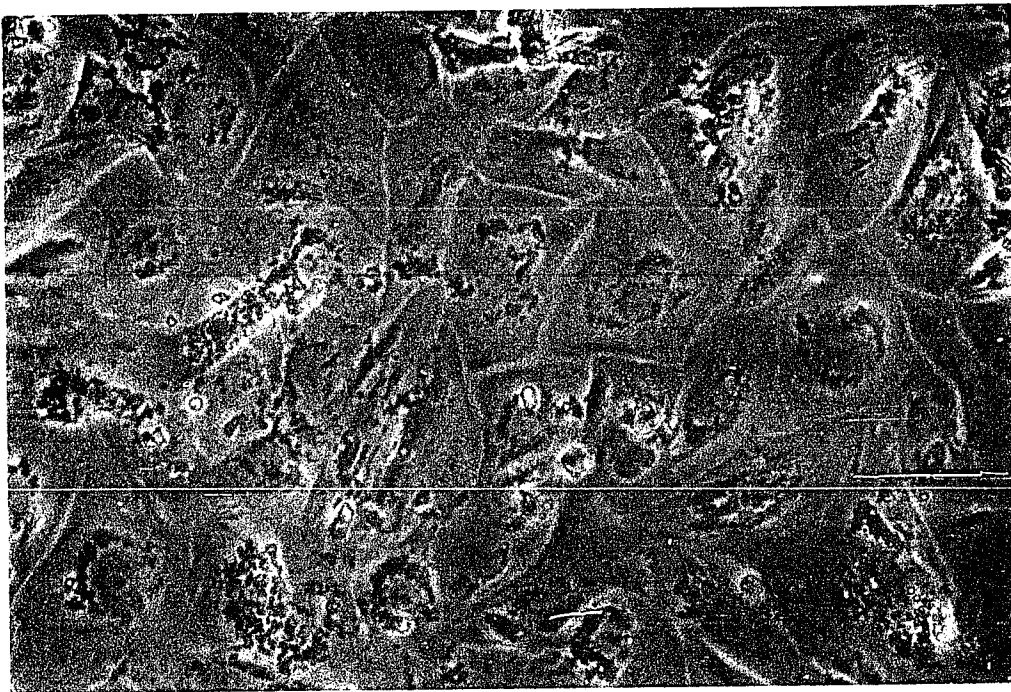
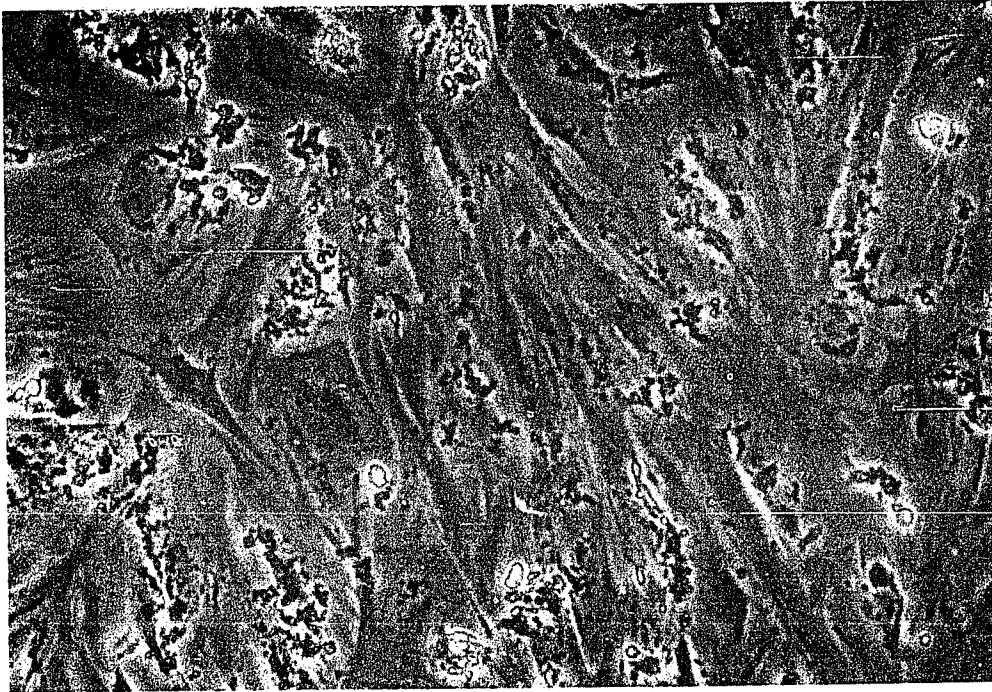
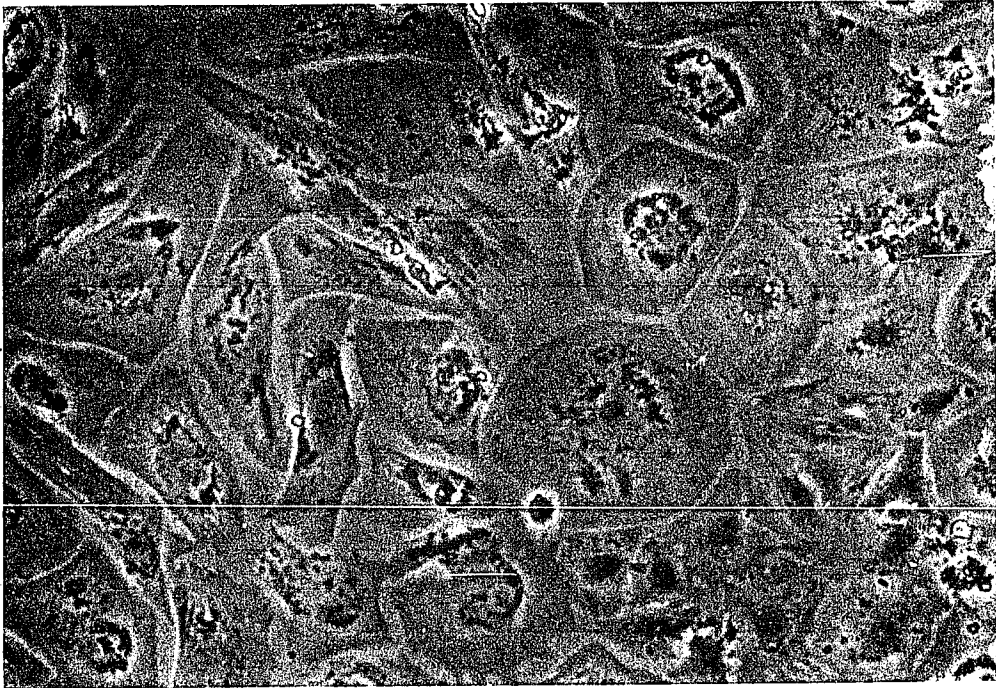
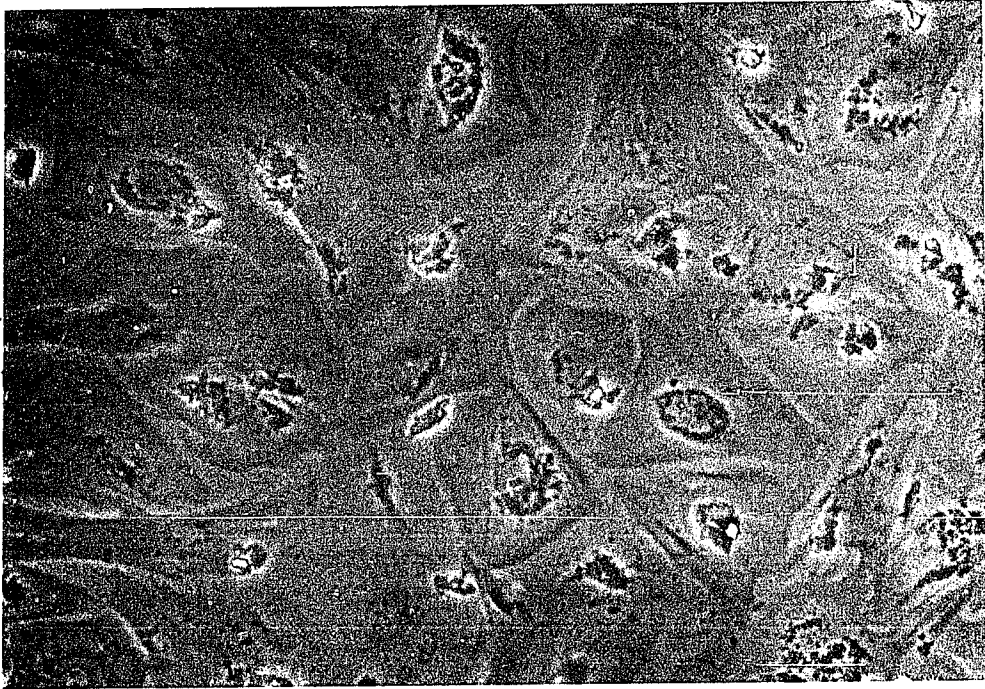


Figure 30. Phase-contrast photomicrograph of bovine luteal cells cultured for 8 days in serum-free medium with the addition of  $\text{PGF}_{2\alpha}$  (10ng/ml). x195.

Figure 31. Phase-contrast photomicrograph of bovine luteal cells cultured for 8 days in serum-free medium with the addition of LH +  $\text{PGF}_{2\alpha}$ . x195.





## DISCUSSION

### Serum vs. Serum-Free Medium

The initial results suggest that the function of bovine luteal cells in tissue culture can best be studied in a serum-free system. Cultured in the presence of serum, the luteal cells are not responsive to LH in any consistent manner. Similarly, Cirillo et al. (1969) reported that bovine granulosa cells cultured in medium containing 10% fetal calf serum were only occasionally responsive to LH and that the conditions for this response were unknown and uncontrollable. The inconsistent response observed in cultures containing serum may be due to the variability between batches of serum. The steroid environment of cultured ovarian cells can inhibit (Magoffin and Erickson, 1982) or enhance (Nimrod, 1981b; Fanjul et al., 1983) gonadotropin-stimulated function, and it is possible that the steroids present in serum would cause variability in response between batches of serum. However, it is unlikely that the steroids in the serum are the sole reason for the lack of responsiveness, since cells cultured in the presence of charcoal-stripped serum exhibited no better response to LH than those in steroid-containing serum (preliminary trial, data not shown).

In contrast, luteal cells cultured in serum-free medium exhibited a consistent pattern of responsiveness to LH. The cultures were highly responsive to LH on day 1 of culture, the response then diminished on days 3 and 5, but returned on days 7-11. This pattern has also been described in primary cultures of adult rat testis cells (Hseuh, 1980). Hseuh postulated that this response pattern might be due to adaptation

to the culture environment or to the presence of two populations of Leydig cells which acquire steroidogenic capacity at different rates. The bovine corpus luteum is known to consist of two cell populations, which differ in their responsiveness to LH (Ursely and Leymarie, 1979; Koos and Hansel, 1981). Since the present experiments employed cultures of mixed large and small luteal cells, it is possible that the two cell sizes contributed to the observed pattern of steroidogenic response to LH, i.e. one cell size may have responded to LH early in the culture period and the other size may have taken until days 7-11 to acquire the ability to respond to LH.

It is likely that insulin, transferrin and hydrocortisone are already present in serum, but their concentrations would be unknown and highly variable. Also, insulin is highly unstable above 0°C, and would probably not last very long in the serum. The cellular responsiveness was not better when these factors were added to the serum-containing medium (Table 2). These results lend support to the hypothesis that the presence of serum in the culture medium inhibits the responsiveness of the luteal cells to LH.

While the ability of the luteal cells to respond to LH only in serum-free medium does not support the work of Gospodarowicz and Gospodarowicz (1972), it is in agreement with the findings of Erickson et al. (1979) and Orly et al. (1980) that rat granulosa cells can respond to FSH only in serum-free medium. Orly et al. (1980) have, however, reported that granulosa cells respond to dbcAMP in the presence of serum. When the luteal cells in the present study were cultured in serum-containing medium, they responded to dbcAMP with a great increase in progesterone production. This suggests that the inhibition imposed

by the serum is at a step in the biosynthetic machinery prior to the accumulation of cAMP.

Savion et al. (1981) reported similar findings in cultures of bovine granulosa cells; in serum-free medium, FSH + dbcAMP increased progesterone to a greater extent than did dbcAMP alone, but there was no difference between these two treatments in serum-containing medium. However, in their system, exposure to FSH alone had no effect on  $P_4$  production in either serum-free or serum-containing medium. They suggested that this was because the stock cultures were passaged for 2-6 weeks in the presence of serum before the serum-free experiments were begun. This would mean that the inhibitory effect of the serum is a chronic one, and remains even after the serum has been removed from the cultures. The nature of the inhibitory substance(s) present in the serum is not known at this time.

The absolute levels of  $P_4$  synthesized over the 11-day culture do not reflect the pattern of responsiveness of the cells to LH. In both serum-containing and serum-free medium, the high levels of  $P_4$  produced during the first day of culture are not maintained in subsequent days. There is a sharp decline in  $P_4$  from day 1 to day 3, then steroid levels drop more gradually until day 11. The addition of tropic hormones can reduce the rate of decline in  $P_4$  but can not maintain the high levels of steroidogenesis found on day 1. Gulyas et al. (1979) reported similar findings with monkey luteal cells in culture. The diminished steroidogenic-activity is probably due to some type of cellular "aging" in culture, with a corresponding decrease in general cellular metabolism. Gulyas and Hodgen (1981) suggested that the  $P_4$  decline was not due to a lack of steroid precursors or hormones in the culture medium, but was

associated with gradual morphologic changes that occur in the cultured cells. After two days in culture the monkey luteal cells became flattened and contained less smooth endoplasmic reticulum than freshly isolated cells. Bovine luteal cells also lose their smooth endoplasmic reticulum in culture (Nord, 1980), which would obviously limit the steroidogenic capacity of the cells.

#### Effects of Prolactin, PGE, and Cell Density

Prolactin is not considered a luteotropic hormone in the bovine, since it does not prolong the estrous cycle or stimulate  $P_4$  synthesis by luteal tissue slices in vitro (Hansel et al., 1973). However, in other species, prolactin usually exerts its luteotropic effects by maintaining overall cellular integrity and enzymatic function. These chronic actions of prolactin would be important in a long-term cell culture system to aid in the maintenance of steroidogenic capacity. Although prolactin sustains  $P_4$  levels slightly above controls, this effect is not significant and could be due to the LH contamination in the prolactin preparation. More importantly, prolactin does not potentiate the acute steroidogenic action of LH, even after prolonged exposure. These results support the conclusion that prolactin has no luteotropic effect in the bovine. Romanoff (1966) has reported that prolactin stimulates  $P_4$  synthesis by isolated perfused bovine ovaries, but extremely high levels of prolactin were used and his results could be due to LH contamination.

Prostaglandin  $E_1$  and  $E_2$  stimulate adenylate cyclase activity and progesterone synthesis by the bovine corpus luteum in vitro (Hansel, et al., 1973; Marsh, 1976), but the long-term in vitro effects of these prostaglandins had not been previously examined. The drop in respon-

siveness to  $\text{PGE}_1$  on day 3 is identical to that seen with LH and dbcAMP (Figs. 3 and 4b). It is possible that the cells need time to adapt to the culture environment, and regain their responsiveness after this acclimation period.  $\text{PGE}_1$  is generally stimulatory throughout the culture period and, therefore, can be used to study hormone-induced  $\text{P}_4$  production in this system.

Hornsby and Gill (1981), using cultured bovine adrenocortical cells, reported that a major determinant of cellular responsiveness to ACTH, but not  $\text{PGE}_1$ , was cell density. The reason for this difference is not clear, but an effect of cell density on responsiveness to hormonal stimulation has also been reported in cultured thyroid cells (Filetti et al., 1981) and human fibroblasts (Pochet et al., 1982). Pochet et al. found that  $\beta$ -adrenergic receptors were decreased at high population densities, and Chow and Poo (1982) noticed a redistribution of cell surface lectin receptors after cell contact in cultured muscle cells. This could be related to the change in membrane microviscosity (becoming less fluid) that occurs in densely populated cultures (Inbar et al., 1977).

In light of these observations, it was expected that cell density would affect the responsiveness of cultured bovine luteal cells to gonadotropin stimulation. No significant differences were observed in the cell density experiments, but this is probably due to the low number of animals used. The highest density culture exhibited the least response to LH on day 1. This could be attributed to the fact that all cultures received the same dose of LH (10ng/ml), and with a greater number of cells in the high density culture there would simply be fewer LH molecules per cell to elicit a maximal response. This does not hold

true throughout the culture period, however, since the second-most dense culture was more responsive than the third-most dense culture. Although it may not be apparent from these experiments, cell density probably does play a role in luteal responsiveness to LH. There is most likely an optimal cell density that would allow the cells to respond to LH, secrete appreciable amounts of steroid, and not be adversely affected by overcrowding in the culture vessel. If this is true, the optimal density for bovine luteal cells remains to be determined.

#### Lipoprotein Experiments

As shown in Figs. 5 and 6, luteal cells cultured in serum-free medium contain far fewer lipid droplets than cells in serum-containing medium. This loss of endogenous cholesterol stores could be a major factor contributing to the decreased output of  $P_4$  observed in the serum-free cultures. Although the addition of LDL and HDL at the onset of the culture did not greatly increase  $P_4$  levels, it demonstrated that neither lipoprotein inhibited the LH response, as had the serum. The greater responsiveness of the cells to LDL and HDL on days 5-9 of culture is probably due to the 3-day "preincubation" prior to lipoprotein addition, which serves to lower the endogenous cholesterol stores in the luteal cells.

Lowering blood lipoprotein levels in rats leads to decreased levels of plasma  $P_4$  (Andersen and Dietschy, 1978; Christie et al., 1979; Azhar and Menon, 1981c), indicating that circulating lipoproteins serve as an important source of cholesterol for ovarian  $P_4$  synthesis. The in vitro stimulation of progesterone production by lipoproteins is consistent with results obtained in cultured rat (Schuler et al., 1979; Schreiber et al., 1980) and bovine (Savion, et al., 1982) granulosa cells. The

Increased  $P_4$  is probably due to increased synthesis, not simply enhanced secretion from the cells, because bovine lipoproteins have been shown to have no effect on secretion of  $P_4$  from luteal tissue in vitro (Condon and Pate, 1981).

Both LDL and HDL were able to stimulate  $P_4$  synthesis, presumably by providing cholesterol substrate. The results of the  $^{14}\text{C}$ -acetate incorporation study indicate that the lipoproteins can alter cellular cholesterol metabolism. Both LDL and HDL caused a decrease in de novo cholesterol synthesis and hence lowered the specific activity of the  $P_4$  being produced. Since cholesterol synthesis was decreased and  $P_4$  synthesis was increased, the cholesterol used for steroidogenesis had to come from either cholesteryl ester stores or from the lipoproteins. As already discussed, the lipid stores in the cells were largely depleted, indicating that lipoprotein-supplied cholesterol was probably the source of sterol for  $P_4$  production. The mechanism by which lipoproteins can regulate cholesterol synthesis is not fully understood, but probably depends primarily on the inhibition of HMG-CoA reductase that occurs when granulosa cells are cultured in the presence of lipoproteins (Savion et al., 1982).

Savion et al. (1982) reported that LDL seemed to be more effective than HDL in promoting  $P_4$  synthesis in cultures of bovine granulosa cells. In the present study, HDL tended to increase  $P_4$  and decrease de novo cholesterol synthesis to a greater extent than LDL. The reason for this discrepancy may be due to a difference between bovine granulosa and luteal cells. However, it would seem that the granulosa cells would readily utilize HDL, because they are exposed to only HDL in situ. HDL is present in the follicular fluid bathing the granulosa cells, but LDL



can not penetrate the basement membrane and, therefore, would not be available for use by the granulosa cells. In the present study, lipoprotein was added to the cultures on the basis of lipoprotein-cholesterol content, whereas Savion et al. added lipoproteins on the basis of lipoprotein-protein. This might also contribute to the observed differences in LDL-HDL effectiveness, since the cholesterol/protein ratios are different in the two molecules.

#### Effects of Indomethacin and Prostaglandin

As shown in Fig. 16 and Table 3, bovine luteal cells in culture are capable of synthesizing prostaglandins. Like progesterone, prostaglandin levels tend to decrease over the culture period. Although the differences were not significant, LH caused a slight increase in all three of the prostaglandins. This is in agreement with the findings of Demers et al. (1973), who demonstrated an LH-stimulated rise in PGF in rat corpora lutea in organ culture. The increase in 6-keto-PGF<sub>1α</sub> is intriguing, because prostacyclin has luteotropic effects on the bovine CL both in vivo and in vitro (Milvae and Hansel, 1980b). It is possible that prostacyclin production could serve to potentiate the tropic effects of LH.

However, LH is able to stimulate P<sub>4</sub> production even when prostaglandin synthesis is lowered (Table 4). Inhibition of prostaglandin synthesis by indomethacin resulted in an increase in LH-stimulated P<sub>4</sub> synthesis in rat and ewe corpora lutea incubated in vitro (Evrard et al., 1978), and indomethacin increased both basal and LH-stimulated P<sub>4</sub> in the present study. It is possible that luteal synthesis of prostaglandin suppresses the ability of the cells to achieve maximal steroidogenesis. Balmaceda et al. (1979) have proposed that an elevated PGF/PGE

ratio within the rhesus monkey CL is associated with luteal regression. If this ratio is lowered with in vivo injections of hCG, the corpora lutea exhibit fewer signs of regression than control corpora lutea from the same day of the cycle (Balmaceda et al., 1981). Rothchild (1981) has speculated that an intraluteal prostaglandin may serve as the universal luteolysin. The ability of indomethacin to greatly increase  $P_4$  in cultured bovine luteal cells, suggests that endogenous prostaglandin synthesis may partially regulate  $P_4$  production in this species.

Addition of  $PGF_{2\alpha}$  to the luteal cell cultures either had no effect or produced a slight stimulation of basal  $P_4$  synthesis. Evrard et al. (1978) and Wright et al. (1980) reported that  $PGF_{2\alpha}$  had no effect on basal levels of  $P_4$ , while  $PGF_{2\alpha}$  stimulated basal  $P_4$  synthesis in other in vitro systems (Speroff and Ramwell, 1970; Hansel et al., 1973; Hixon and Hansel, 1979). The ability of  $PGF_{2\alpha}$  to influence basal levels of  $P_4$  is probably variable and dependent on the metabolic state of the cell. Response to  $PGF_{2\alpha}$  might be modulated by the steroidogenic potential of the cell, intracellular levels of steroids and/or prostaglandins; intrinsic mechanisms regulating  $PGF_{2\alpha}$  receptors, or other changing metabolic events within the cell.

#### Mechanism of Action of $PGF_{2\alpha}$

Thomas et al. (1978) reported that  $PGF_{2\alpha}$  stimulated basal  $P_4$  synthesis in a 2 hour culture of rat luteal cells, but completely inhibited LH-stimulated steroidogenesis. This suggests that simultaneous stimulation by another hormone may influence  $PGF_{2\alpha}$  action. In the present study,  $PGF_{2\alpha}$  inhibited LH-stimulated  $P_4$  on days 3-11, although it had no effect on basal steroidogenesis (Figures 17 and 18). However,  $PGF_{2\alpha}$  was not able to overcome the LH stimulation during the first 24

hours of culture. Henderson and McNatty (1977), using cultures of bovine granulosa cells, reported that  $\text{PGF}_{2\alpha}$  had an inhibitory effect if added at the start of the culture, but not if added later in the culture period. Cultured granulosa cells initially produced very low levels of  $\text{P}_4$ , and these levels increase as the culture progresses and the cells undergo "luteinization". These authors suggested that  $\text{PGF}_{2\alpha}$  uptake and subsequent luteolytic action was inversely related to  $\text{P}_4$  production. This would agree with the results reported here, since  $\text{P}_4$  production is much greater on day 1 in luteal cell cultures, and  $\text{PGF}_{2\alpha}$  does not inhibit LH-stimulated steroidogenesis at this time. Unfortunately, Henderson and McNatty used 20% calf serum in their granulosa cell cultures and could get no LH-response, so it is not possible to distinguish between  $\text{PGF}_{2\alpha}$  inhibition of basal or LH-stimulated  $\text{P}_4$  in their system.

The exact mechanism by which  $\text{PGF}_{2\alpha}$  inhibits LH-stimulated steroidogenesis is not clear. It is probably not due to a loss of LH receptors, since  $\text{PGF}_{2\alpha}$  does not reduce gonadotropin uptake in the rat CL in vitro (Behrman et al., 1978; Pang and Behrman, 1981). To determine if the  $\text{PGF}_{2\alpha}$  inhibition occurs prior to or at the level of the adenylate cyclase molecule, cholera toxin and forskolin were used as stimulatory agents. Cholera toxin stimulates adenylate cyclase by direct interaction with the regulatory subunit of the enzyme. Incubation of rat luteal cells with cholera toxin produces a dose-dependent increase in  $\text{P}_4$  production (Azhar and Menon, 1981c). Forskolin is a natural diterpene that acts directly on either the catalytic unit of adenylate cyclase (Seamon and Daly, 1981) or some other component of the enzyme (Stengel et al., 1982). It potentiates ACTH-induced steroidogenesis in adrenal cells (Moriwaki et al., 1982) and stimulates cAMP

accumulation in thyroid membranes (Fradkin et al., 1982).

Both cholera toxin and forskolin were highly stimulatory in cultures of bovine luteal cells (Figures 19-24). As was the case with LH,  $\text{PGF}_{2\alpha}$  was not able to inhibit the cholera toxin or forskolin stimulation on day 1, but significantly depressed any increase in  $\text{P}_4$  on days 3-11. The ability of  $\text{PGF}_{2\alpha}$  to inhibit this stimulation indicates that the site of action of  $\text{PGF}_{2\alpha}$  is not before the adenylate cyclase molecule, but must lie either at the level of the cyclase or beyond this point in the steroidogenic pathway.

If  $\text{PGF}_{2\alpha}$  was acting directly on the adenylate cyclase molecule, addition of dbcAMP would be expected to overcome the prostaglandin inhibition. This is difficult to determine from the present study, because the dbcAMP stimulation was not significant after day 1 in this set of experiments. However, on days 5 and 9, the dbcAMP +  $\text{PGF}_{2\alpha}$  - treated cultures produced somewhat lower levels of  $\text{P}_4$  than those exposed to dbcAMP alone. This would suggest that  $\text{PGF}_{2\alpha}$  can inhibit steroidogenesis at a point distal to the accumulation of cellular cAMP.

These results disagree with those of Thomas et al. (1978), who reported  $\text{PGF}_{2\alpha}$  inhibited the LH-stimulated accumulation of cAMP, and addition of dbcAMP to the rat luteal cells overcame the PG inhibition of LH-stimulated  $\text{P}_4$  production. Dorflinger and Behrman (1979) also found that dbcAMP could overcome the PG inhibition of cholera toxin stimulation in rat luteal cells. Both of these studies employed 2 hour incubations of rat luteal cells. It is possible that  $\text{PGF}_{2\alpha}$  exerts an inhibitory effect at a site after cAMP, but that longer exposure to the prostaglandin is required for this to occur. Also, a small component of inhibition remained even after the addition of dbcAMP in the study by

Dorflinger and Behrman, and while  $\text{PGF}_{2\alpha}$  completely blocked cholera toxin-dependent progesterone secretion, cholera toxin-dependent cAMP accumulation was only partially reduced. These workers concluded that cholera toxin stimulated some adenylate cyclase moieties that were not linked to  $\text{P}_4$  synthesis and were not inhibited by  $\text{PGF}_{2\alpha}$ . However, it seems equally plausible that  $\text{PGF}_{2\alpha}$  produced a lesion in the biosynthetic pathway at a point distal to cAMP.  $\text{PGF}_{2\alpha}$  might also have multiple sites of action in luteal cells, which would further insure the onset of functional luteolysis.

The work of Jordan (1981) supports the concept that  $\text{PGF}_{2\alpha}$  may inhibit steroidogenesis at a point beyond cAMP in rat luteal tissue. Khan and Rosberg (1979) incubated rat luteal membranes and whole cells with  $\text{PGF}_{2\alpha}$  and examined subsequent stimulation of adenylate cyclase by LH. Addition of  $\text{PGF}_{2\alpha}$  to isolated membranes did not change the stimulation of adenylate cyclase by LH, whereas incubation with  $\text{PGF}_{2\alpha}$  in whole cells reduced LH-stimulated adenylate cyclase. Although not discussed by these authors, these results may suggest that  $\text{PGF}_{2\alpha}$  can initiate (or suppress) an intracellular event that would subsequently influence enzyme activity in the plasma membrane.

$\text{PGF}_{2\alpha}$  also inhibited indomethacin-stimulated  $\text{P}_4$  production in the bovine luteal cell cultures. If indomethacin increases  $\text{P}_4$  solely through its prostaglandin synthesis inhibiting properties, then the addition of  $\text{PGF}_{2\alpha}$  may have replaced the endogenous prostaglandins and lowered  $\text{P}_4$ . Since intraluteal prostaglandin synthesis would have been blocked in the presence of indomethacin,  $\text{PGF}_{2\alpha}$  did not inhibit the indomethacin-induced rise in  $\text{P}_4$  through a stimulation of prostaglandin synthesis. However, this does not preclude the possibility that  $\text{PGF}_{2\alpha}$

might block LH-stimulated  $P_4$  by inducing endogenous prostaglandin synthesis. To answer this question, the ability of  $PGF_{2\alpha}$  to inhibit LH-stimulated  $P_4$  in the presence of indomethacin must be examined.

#### Morphology of Hormone-Treated Luteal Cells

The responsiveness of bovine luteal cells cultured in serum-free medium to exogenous hormones is further exemplified by the morphological changes apparent in these cells. Gonadotropin-stimulation causes the cells to become more spherical and separated, and the redistribution of lipid droplets implies that cytoskeletal rearrangements have occurred. It is interesting to find that  $PGF_{2\alpha}$  induces these same changes, and they are still seen when  $PGF_{2\alpha}$  and LH are combined, although steroidogenesis is not exceeding control levels in these cultures. Perhaps the luteal cells respond morphologically to many signals in the same manner, even if these signals ultimately produce different biochemical results.

At the level of the light microscope, it is difficult to distinguish between an LH- and a  $PGF_{2\alpha}$  - treated culture. It would be intriguing to examine the ultrastructure of these cells to determine if LH and  $PGF_{2\alpha}$  have different effects on the cell surface or subcellular organelles of cultured luteal cells.

## CONCLUSIONS

1. From these studies it is concluded that the presence of serum in the cell culture medium diminishes the responsiveness of bovine luteal cells to LH, and that the inhibition imposed by the serum is at a step prior to the increase in cellular cAMP.
2. Serum lipoproteins, both LDL and HDL, are able to enhance progesterone production by cultured bovine luteal cells.
3. LDL and HDL can regulate de novo sterol synthesis and incorporation of acetate into progesterone by cultured bovine luteal cells, supporting the hypothesis that lipoproteins supply luteal cells with a source of cholesterol to be used as substrate for steroidogenesis.
4.  $\text{PGF}_{2\alpha}$  can inhibit LH-, cholera toxin-, and forskolin-stimulated  $\text{P}_4$  production after day 1 of culture. The  $\text{PGF}_{2\alpha}$ -induced lesion in the steroidogenic pathway may be at the adenylate cyclase molecule, at a point distal to the accumulation of cAMP, or both.
5. The stimulation of steroidogenesis by indomethacin can be overcome by the addition of  $\text{PGF}_{2\alpha}$ .

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## APPENDIX

## APPENDIX A

### Radioimmunoassay of Progesterone

#### Assay:

1. Make standard progesterone for standard curve.
  - Defrost 2 vials standard stock progesterone which contains 10 ng/ml.  
Remove 1 ml from each and place into a 25 ml glass-stoppered flask. Add 18 ml ethanol (absolute) (a 1:10 dilution); resulting solution will be 1.0 ng/ml.
  - Amounts for standard curve are:  
2.0, 1.0, 0.4, 0.2, 0.1, 0.06, 0.02 ng/ml  
To obtain these amounts simply pipette 2.0, 1.0, 0.4, 0.2, 0.1, 0.06, 0.02 ml, respectively, into assay tubes.
  - \*For each assay there are two standard curves, one before the samples and one after the samples; in addition, each point on the curve is done in duplicate, then averaged. Thus, there is a total of four assay tubes for each point (two tubes for each curve).  
Sample determinations are read from the average of the two curves.
2. Dry down all standards under air in a 40°C water bath.
3. Pipette 0.1 ml of sample into 2 assay tubes.
4. Add 100 ul assay buffer to all sample tubes and 200 ul assay buffer to standard curve tubes.
  - 950 ul assay buffer to total count tubes.
  - 200 ul assay buffer to non-specific binding tubes.
5. Place rack of assay tubes into ice bath.
6. Mix antiserum with magnetic stirring rod. Pipette 100 ul antiserum into all standard and sample tubes except total counts and NSB (non-specific binding), vortex.
7. Pipette 100 ul assay tracer into all tubes, vortex.  
Cover with tin foil and incubate at 4°C for at least 4 hours (preferably overnight).

#### Separation of Bound and Free Progesterone:

1. At end of incubation transfer rack of tubes into a fresh ice bath and add 750 ul cold dextran-coated charcoal. \*Except to total counts tubes. Charcoal should be kept in an ice bath and under constant stirring while adding to tubes.
  - \*Add the charcoal as rapidly as possible and not to more than 30-40 tubes at one time to avoid stripping.
2. Promptly vortex each tube briefly and place into centrifuge immediately. Time from the start of adding charcoal, to starting the centrifuge should not exceed 1 minute.
  - Centrifuge 10 min. at 1700 xg in refrigerated centrifuge.
3. Decant supernatant fraction of each tube into numbered scintillation insert vials.  
Do not disturb charcoal pellet at the bottom.

4. Add 4 ml counting cocktail, vortex, cover vials with tin foil for at least 4 hours and then count for 1 minute. \*Charcoal suspension picks up free progesterone leaving the bound progesterone on the antibody in solution to be counted. \*Samples must sit for at least 4 hours to allow the cocktail to extract the  $^3\text{H}$ -progesterone out of the sample.

Preparation of Solutions:

1. Assay Buffer Do not store for more than 2 weeks

$\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	5.38 g
$\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	16.35 g
$\text{NaCl}$	9.0 g
Gelatin	1.0 g
Na Azide	1.0 g

Add 900 ml 10 megohm  $\text{H}_2\text{O}$  and mix on a warm magnetic stirrer and adjust pH to 7.0 with 1.0 N NaOH. Adjust volume to 1 liter with 10 megohm  $\text{H}_2\text{O}$ .

2. Charcoal Suspension Do not store for more than 2 weeks

20 mg Dextran T-70

200 mg prewashed Norit A neutral charcoal

Add 100 ml assay buffer and magnetically stir approximately 20 minutes at high speed while placed in an ice bath.

3. Antiserum

Always dilute with assay buffer. The activity of the antibody determines amount of dilution. Make up final dilution the day of the assay.

4. Assay Tracer

Pipette 20  $\mu\text{l}$  tritiated progesterone( $^3\text{H-P}_4$ ) into a counting vial. Dry down. Add 10 ml assay buffer. Vortex 30 seconds at high speed. Place in  $40^\circ\text{C}$  water bath for 20 minutes. Refrigerate before using.

5. Counting Cocktail

1.32 g PPO

1 liter toluene

Stir on magnetic stirrer for approximately 15 minutes.